Antiproliferative effect of a novel synthesized carbazole compound on A549 lung cancer cell line

By

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in the

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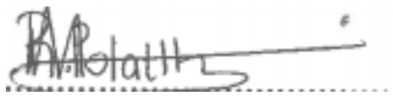
DECLARATION

This dissertation represents the original work by the author and has not been submitted in any form to another university. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Prof. A.A. Chuturgoon and Dr. Alisa Phulukdaree.



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Miss R. P. Molatlhegi

ABSTRACT

Increased death rates due to lung cancer have necessitated the search for potential novel anticancer compounds such as carbazole derivatives. Carbazoles are aromatic heterocyclic compounds with anticancer, antibacterial and anti-inflammatory activity. The study investigated the ability of the novel carbazole compound (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one (ECAP) to inhibit the proliferation of lung cancer cells and its mechanism of action. ECAP was synthesized as a yellow powder with melting point of 240-247 °C. The 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lipid peroxidation and comet assays were used to assess the anti-proliferative effects of the compound on A549 lung cancer cell line. Protein expression was determined using western blots, apoptosis was measured by luminometry for caspase-3/7, -8 and -9 and flow cytometry was used to measure phosphatidylserine externalisation.

ECAP induced a p53 mediated apoptosis of lung cancer cells by significantly down-regulating the expression of antioxidant defense proteins, Hsp70 ($p < 0.02$) and Bcl-2 ($p < 0.0006$), thereby up-regulating reactive oxygen species (ROS) production. This resulted in DNA damage ($p < 0.0001$) and subsequent up-regulation of Bax and caspase activity consequently inducing apoptosis of lung cancer cells. These results demonstrate the potential anticancer effects of ECAP on cultured lung cancer cells. However, further investigation and characterization is required to fully understand the possible use of carbazole compound (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one as potential lung cancer treatment.

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PRESENTATIONS

Anticancer Properties of a novel carbazole compound on A549 lung cancer cell line.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndromes
Apaf-1	Apoptotic protease activating factor-1
ARE	Antioxidant response elements
ATM	Ataxia-telangiectasia mutated
BCA assay	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma/leukaemia-2
CARD	Caspase-activating recruitment domain
CDKs	Cyclin-dependent kinases
Chk2	Checkpoint kinase 2
COPD	Chronic Obstructive Pulmonary Diseases
CRDs	Cysteine-rich domains
DD	Death domain
DEDs	Death effector domains
DISC	Death-inducing signaling complex
ECAP	(Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one
ETC	Electron Transport Chain
FADD	Fas-associated death domain
FC	Fold change
G_0 phase	Arrested or quiescent phase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl radical
HRP	Horse radish peroxide

Hsp70	Heat shock protein 70
M phase	Mitosis/ meiosis phase
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MDM2	Murine double minute 2
NES	Nuclear export signal
NLS	Nuclear localization signal
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung cancer
O^{-}_{2}	Superoxide anion
PS	Phosphatidylserine
ROS	Reactive oxygen species
S phase	Synthesis phase
SA	South Africa
SAPK	Stress-activated protein kinase
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SV40	Simian virus 40
TB	Tuberculosis
TBARS	Thiobarbituric acid reactive substances
TEMED	Tetramethylethylenediamine
TNFR1	Tumor necrosis factor receptor type 1
TRADD	TNF-related (TNFR) associated death domain

wt p53	Wild-type p53

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INTRODUCTION

Cancer is the second leading disease after cardiovascular diseases worldwide, accounting for high mortality rates (Bello *et al.*, 2011). According to Globocan the global new cancer cases and cancer related deaths increased from about 12,7 million and 7,6 million in 2008 to 14,1 million and 8,2 million in 2012 respectively. Lung cancer is the main cause of cancer related deaths worldwide with more than million deaths each year (Jemal et al., 2004). Most of global cancer cases are reported in developing countries, with a high mortality rate of 63% (Ferlay *et al.*, 2010). An increase in cancer cases such as lung cancer in developing countries such as South Africa (SA) are associated with infectious diseases like Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndromes (AIDS) and tuberculosis (TB) (Bello *et al.*, 2011). During 2006 alone about 4 525 lung cancer related deaths were reported in SA. In addition increased lung cancer cases in SA are also attributed to other factors such as smoking, environmental pollutants, occupational exposure and changing lifestyles (Bello *et al.*, 2011).

Reactive oxygen species (ROS) are one of the major causes of malignancy. They are products of oxygen metabolism in cells and their functions range from cell signaling, homeostasis and antimicrobial effects (Uttara *et al.*, 2009). Mammalian cells are continually exposed to ROS generated both extrinsically and intrinsically (Pelicano *et al.*, 2004). Chronic and persistent exposure to high ROS levels induces DNA, protein and lipid damage, and can result in induction of diseases such as cancer (Waris and Ahsan, 2006).

Cellular homeostasis in multicellular organisms is strictly maintained through regulation of cell proliferation and cell death by the cell cycle and apoptosis.

The cell cycle is a tightly regulated process under the control of specialized regulatory molecules such as cyclins and cyclin dependent kinases (CDK). However, mutation of tumor suppressor genes like p53 and elevated levels of ROS may allow for unregulated growth leading to cancer development. Mammalian cells contain defense systems to regulate oxidative damage, and these include proteins such as superoxide dismutase (SOD), nuclear factor erythroid 2-related factor 2 (Nrf2) and heat shock protein 70 (Hsp70) (Scherz-Shouval and Elazar, 2011, Whitacre *et al.*, 1999, Suzuki *et al.*, 2013) .

Different therapies including chemotherapy, surgery and radiotherapy have been used for cancer treatment. However, chemotherapy and radiotherapy lack specificity therefore healthy cells can also be harmed during treatment, also the survival rates for lung cancer patients undergoing surgery is poor (Cavendish, 2008). There are no effective treatment drugs for lung cancer, hence there is need for more potent anti-lung cancer drugs. The challenge still is the ability to develop highly effective drugs specific for lung cancer with slight or no side effects on normal cells.

Heterocycles are aromatic molecules joined in a ring that consists of at least one element other than carbon. These compounds have become an important class of organic compounds for research because of their numerous medical and agricultural applications (Liu and Larock, 2007).

Carbazoles are aromatic heterocyclic organic compounds, with a tricyclic structure comprising of two benzene rings each fused onto a five-structured nitrogen-containing ring (Liu and Larock, 2007, Nandy *et al.*, 2014).

These synthetic or natural products function through interaction with the DNA; they damage DNA resulting in inhibition of synthesis of new DNA or RNA (Roy *et al.*, 2005). Carbazoles and their derivatives inhibit cancer growth by intercalating into DNA, inhibiting DNA topoisomerase II activity, as well as through covalent DNA adducts formation, which are mediated through oxidation by cytochrome P_{450} and peroxides (Nandy *et al.*, 2014).

Different natural and synthetic carbazole derivatives including elipticine, elivacine, elliptinium acetate, mahanimbine, eukonine, koenoline and rebaccamycin have been reported to have antineoplastic activity (Nandy *et al.*, 2014). Based on their reported anti-tumour, antibacterial and anti-inflammatory activities, different synthetic carbazole analogs have been synthesized from naturally occurring carbazoles (Liu and Larock, 2007, Nandy *et al.*, 2014). Carbazoles and their derivatives are increasingly being targeted for potential use in cancer treatment owing it to their large π-conjugated system, which make it easy to introduce different functional groups into the rigid carbazole ring (Nandy *et al.*, 2014).

AIM AND OBJECTIVES

The primary goal of the study was to investigate the anti-proliferative properties of a novel synthesized carbazole compound (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one (ECAP) on A549 lung cancer cell line.

The objectives were:

1. To determine the cytotoxicity of ECAP using MTT assay.
2. To examine the genotoxic effect of ECAP on A549 cells using comet assay.
3. To establish the effect of ECAP on ROS production in A549 lung cancer cell line.
4. To investigate the effect of treating A549 lung cancer cells with ECAP on the expression of anioxidant, apoptotic and anti-apoptotic associated proteins using western blot analysis and flow cytometry.

CHAPTER ONE

1.1. LITERATURE REVIEW

1.1.1. Cancer biology

The work of previous scientists including Mendel, Darwin, Watson and Crick has provided us with a foundation to understand different fields of molecular biology, genetics as well as medical and cancer research (Weinberg, 2014). It all started in the early 19th century when Gregor Mendel established the basic rules of genetics through characterization of pea plants. It was through the light of his principles of genetics and mutation that further helped with explaining the Darwinian principles of evolution and how mutations are responsible for the change in genetic information of the gene, thereby altering the characteristics of the affected allele (Weinberg, 2014).

These mutant alleles can then be passed from parent to the offspring; however mutations found in the somatic cells are not transmitted to the offspring and can give rise to a number of diseases such as cancer. Tumour cells arise from many specialized cells in the human body. The formation of the human body is governed by the cell cycle and division which give rise to tissues; tissues in turn results in the formation of organs which form the human body (Figure 1) (Almeida, 2010).

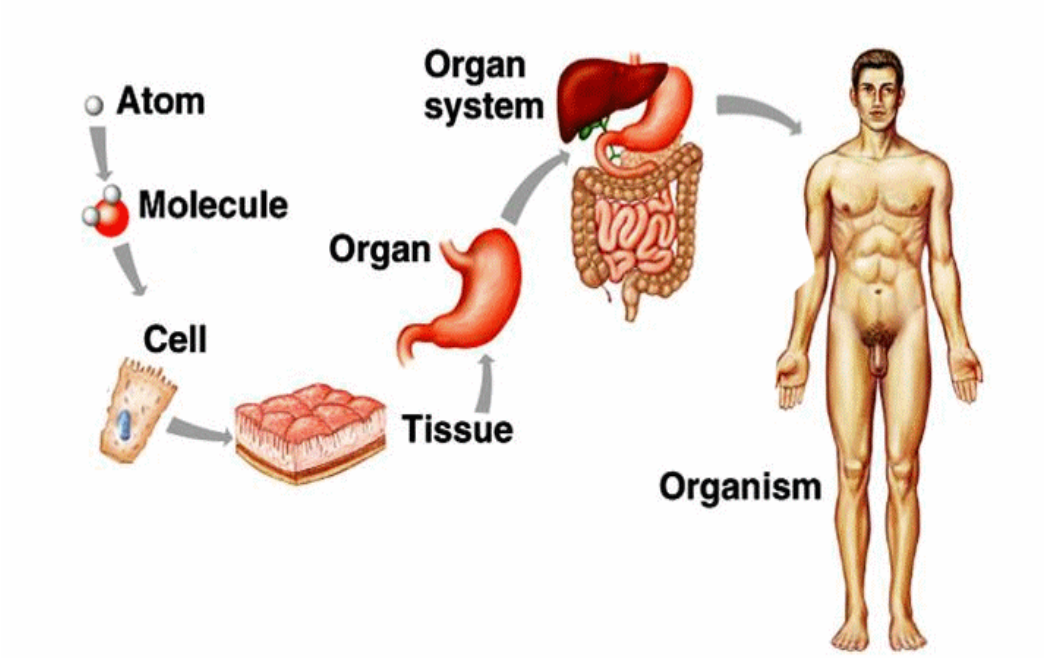


Figure 1: The seven hierarchal levels of organisation forming the body (http://anaphysiology.blogspot.com/)

1.1.1.1. Cell cycle

Cell division is an essential component for the development and growth of organisms. It is governed by a series of events known as the cell cycle; a process tightly regulated by regulatory proteins known as cell-cycle control system, which ensure that the integrity of the organism is maintained (Park and Lee, 2003, Novák and Tyson, 2004). Loss of cell cycle regulation can result in uncontrolled cell growth, the main characteristic essential for cancer formation (Almeida, 2010). The main function of the cell cycle is to ensure accurate genomic duplication and chromosome segregation into each daughter cell (Almeida, 2010, Alberts *et al.*, 2008).

The eukaryotic cell cycle consists of G_1 and G_2 Gap phases, DNA synthesis phase (S phase), M phase (mitosis/ meiosis) and the arrested or quiescent phase (G_0 phase) Figure 2 (Park and Lee, 2003, Tyson and Novak, 2001). The G_1, S and G_2 phases collectively form the interphase, whereby the cell function normally and prepare itself for division.

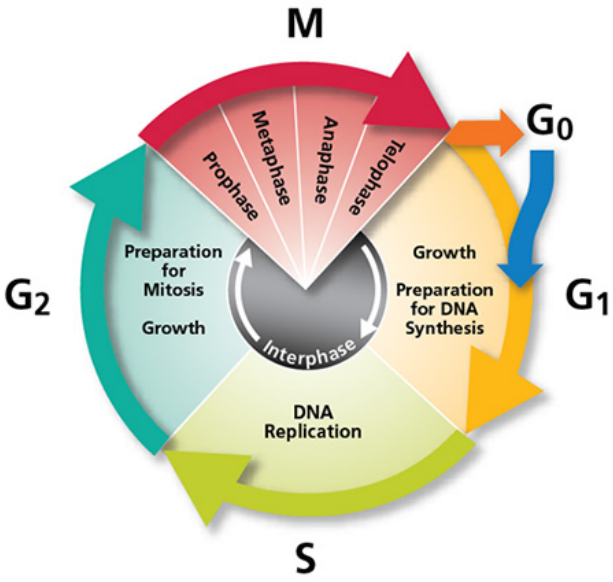


Figure 2: The five phases of the cell cycle

(http://www.bdbiosciences.com/research/apoptosis/analysis/index.jsp)

Depending on the environment and cell growth signals, cells may remain in G_1 or may leave the cell cycle temporarily or permanently and enter the G_0 phase. However, during cell division, the cell enters G_1 and progress to the S phase for chromosome replication (Almeida, 2010, Alberts *et al.*, 2008). The cell then moves to G_2 phase where it increases its size and develops more components preparing itself for division. If the DNA is correctly synthesized it progresses to the M phase where it divides producing two daughter cells.

The newly synthesized daughter cells will then enter the G_1 phase. On average the human cell cycle process takes 12-24 hours of which approximately 30 minutes to one hour is spent at the M phase (Almeida, 2010).

1.1.1.1.1. Regulation of the cell cycle

Each stage of cell cycle consists of checkpoints whereby the cycle is temporarily halted until certain conditions are met. The cell cycle process is dependent on activation and inactivation of cyclin-dependent kinases (CDKs), the small serine/threonine protein kinases whose activity phosphorylates the intracellular proteins essential for the regulation of the cell cycle (Almeida, 2010, Alberts *et al.*, 2008, Park and Lee, 2003, Rastogi and Mishra, 2012). CDKs activity is regulated by tightly binding to the cyclins (Alberts *et al.*, 2008).

The four classes of cyclins are: (1) G_1/S-cyclins which activates the CDKs in the late G_1 phase initiating the start of cell cycle and their levels drop in the S-phase, (2) S-cyclins, which bind to the CDKs activating chromosome replication. S-cyclin remains active until mitosis. (3) M-cyclins activate CDKs, which initiate the entry into the M-phase at the G2/M checkpoint, and the (4) G_1-cyclins that regulate the activity of G_1/S cyclins. CDK activity is suppressed by inhibitory phosphorylation and CDK Inhibitory Proteins (CKIs) (Alberts *et al.*, 2008). In cancer cells, genes encoding CDKs, CDK-activating enzymes, cyclins and checkpoint proteins are mutated thus resulting in overexpression of proteins such as CDKs and cyclins (Vermeulen *et al.*, 2003).

CDK and cyclin overexpression is evident in various human cancers (Park and Lee, 2003). Overexpression of cyclin A and cyclin E has been reported in lung carcinoma (Dobashi *et al.*, 1998).

1.1.2. Cancer formation

The word cancer was introduced around 460 – 370 BC by Hippocrates the Greek physician, and it is used to describe the disease characterized by unregulated proliferation (Figure 3) and the ability to spread into other body parts (Almeida, 2010). Over hundred different types of cancers have been identified and they are classified into carcinomas, sarcomas, leukaemias and lymphomas depending on the tissue from which they originate (Alberts *et al.*, 2008). Majority of human cancers (about 90%) are carcinomas, which are cancers arising from epithelial cells. Sarcomas are less common and they arise from connective tissue, bone, fat or muscle, while leukemias and lymphomas arise from white blood cells (Alberts *et al.*, 2008, Almeida, 2010).

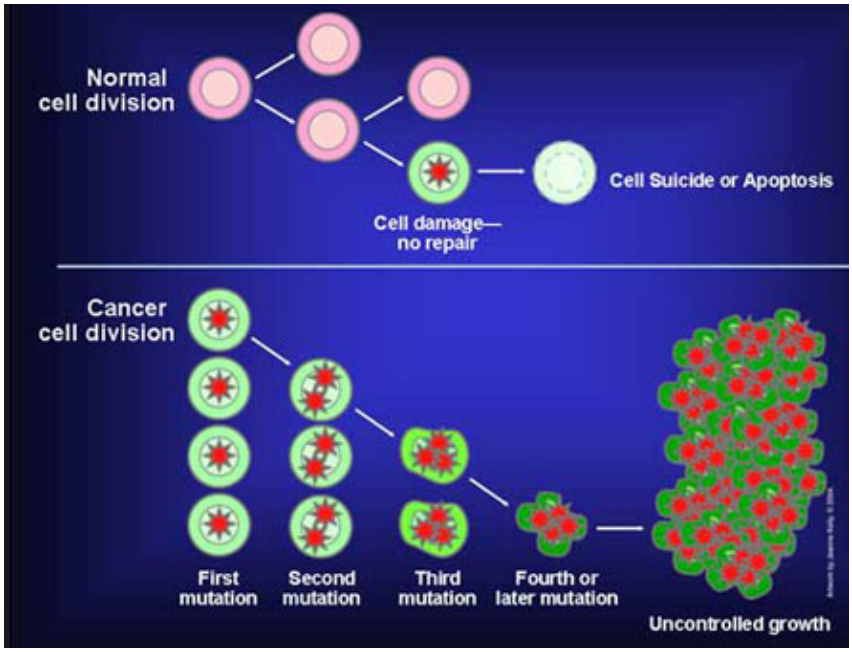


Figure 3: An overview of cancer formation from loss of normal growth control (http://www.petcancercenter.org/About_Cancer_Main_Page.html)

1.1.3. Cancer cases

Majority of global cancer cases are reported in developing countries such as South Africa (SA), with 63% mortality rate (Ferlay *et al.*, 2010). This is due to poverty, lack of knowledge, poor health facilities, lack of trained health professionals and late diagnosis. Majority of cancers in developing countries are diagnosed at an advanced stage due to late detection, thus account for poor survival after diagnosis. Commonly reported cancers include breast (1,7 million, 11.9%), lung (1,8 million, 13%) and colorectum (1.4 million, 9.7%).

1.1.3.1. Lung cancer

Lung cancer is an important form of cancer worldwide accounting for 1.4 million (18.2%) deaths (Ferlay *et al.*, 2010).

Lung cancer is a malignant tumour of the lung characterized by uncontrolled cell proliferation in the lung tissues. The two main types of lung cancer include the non-small cell lung cancer (NSCLC) and the small cell lung cancer (SCLC) (Almeida, 2010). NSCLC accounts for about 85% of lung cancers (Parsons *et al.*, 2010). NSCLC is further characterized into squamous cell carcinoma which is the commonly known type of NSCLC originating from the bronchial tubes, adenocarcinoma found in the mucus producing lung glands, bronchio-alveolar carcinoma which starts close to the air sacs and large-cell undifferentiated carcinoma which forms from near the outer edges of the lung.

1.1.3.1.1. Risk factors associated with lung cancer development

Lung cancer development is attributed to by many factors such as DNA damage, epigenetic changes which can invade cellular processes including proliferation, DNA repair and apoptosis as well as family history of the disease. However, tobacco smoking is reported to be the main cause of lung cancer and it accounts for 80-90% of lung cancer cases (Figure 4) (Almeida, 2010). Lung cancer development can also be caused by environmental pollutants such as exposure to radon, an odourless, invisible radioactive gas capable of inducing DNA damage as well as asbestos responsible for development of mesothelioma, a form of lung cancer originating in the lining of the lung (Almeida, 2010).

Developing countries are reported to have a correlation between TB, HIV, smoking and chronic obstructive pulmonary diseases (COPD) (Acehan *et al.*, 2002).

There is also an increasing number of parasitic and bacterial infections which are associated with cancer development. The relationship between lung cancer and TB has been associated with the ability of lung cancer treatment to activate latent *M tuberculosis* (Marais *et al.*, 2013). There is an increase in lung cancer risk in HIV infected young people with a long history of tobacco smoking (Remick, 1992, Slee *et al.*, 1999).

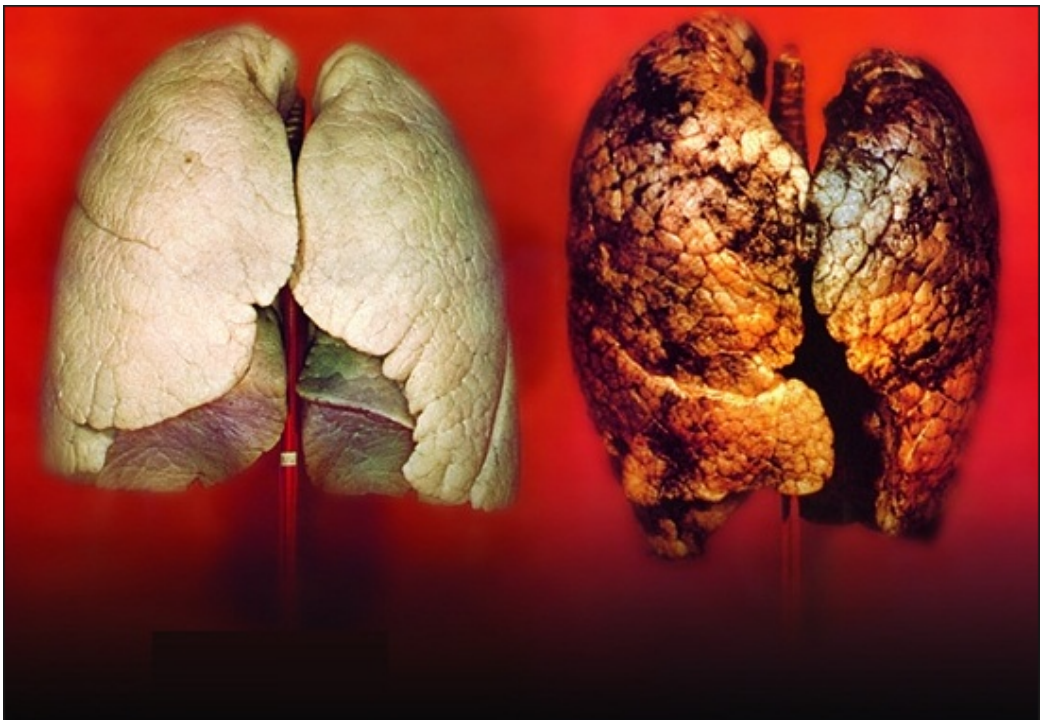


Figure 4: An image of (a) health lung and (b) cancerous lung (http://cancerssymptoms.org/lung-cancer)

1.1.3.1.2. Types of treatments

The three standard cancer treatment strategies include surgery, chemotherapy and radiation. Surgery is a commonly used technique at an early stage of cancer and it involves the removal of the primary tumour.

Early detection of lung cancer is suggestive of lobectomy, which involves the removal of the entire lobe.

Other surgical techniques for lung cancer treatment include wedge resection or segmentectomy, that is used to remove a portion of the malignant tissue in a lobe and pneumonectomy which, is the removal of the entire lung (Almeida, 2010).

Chemotherapy is a standard form of cancer therapy, which is used to prevent cancer cells from multiplying. Typical chemotherapy drugs used for NSCLC include carboplatin or cisplatin, which is used in combination with paclitaxel (Almeida, 2010). However, chemotherapy can be harmful to healthy cells resulting in serious side effects (Cavendish, 2008); as a result there is a need for development of more target specific and effective anti-cancer drugs.

Radiotherapy is another form of lung cancer treatment which uses high energy radiation targeted at cancerous tissue. The high energy radiation permanently damages the DNA of cancerous cells resulting in their death (Cavendish, 2008). Radiotherapy can be performed from inside the body (internal radiotherapy) or from outside the body (external radiotherapy). Radiotherapy can be used together with surgery and/or with chemotherapy to reduce the tumour size relieving pain and bleeding, as well as for opening air passages to enhance air flow in the lung (Almeida, 2010). The side effects of radiotherapy include sore skin, tiredness, hair loss and it takes time for the beneficial effects to be apparent.

Nonetheless, just like chemotherapy, radiation therapy is not specific and it damages both cancer and non-cancer cells (Cavendish, 2008).

Therefore there is need for the discovery of new therapeutic drugs for lung cancer, which are effective, specific and with less side effects.

1.1.4. Reactive Oxygen Species

Reactive oxygen species (ROS) are oxygen containing free and non-radical molecules including: hydroxyl radical ($HO^{\cdot}$), superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2) and hypochlorite anion (OCl^{-}). They are extremely unstable and react readily with other molecules. They are formed through a number of mechanisms, which include cellular respiration, and also they are synthesized by phagocytic cells like neutrophils and macrophages (Figure 5) (Li *et al.*, 2011, Uttara *et al.*, 2009). Their production is essential for conducting cellular biochemical activities including signal transduction, muscular proliferation, gene transcription and killing of microbes (Uttara *et al.*, 2009). Humans are also exposed to ROS from environmental factors including pollution and cigarette smoke.

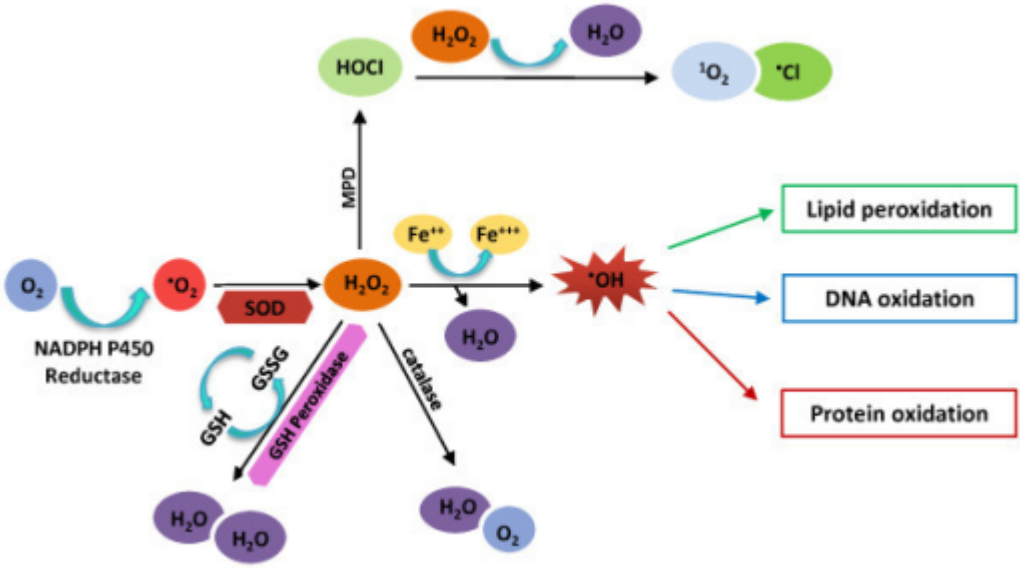


Figure 5: The effect of ROS overproduction (Castello *et al.*, 2010).

1.1.4.1. Cellular defense against ROS

ROS are strong oxidants and can damage cells structures and other cellular molecules. Eukaryotic cells possess antioxidant defense systems, which include superoxide dismutase, catalases, glutathione peroxidase and glutathione reductase that are known to reduce free radicals to less harmful species in order to ensure cellular homeostasis. The anti-oxidants provide electrons to free radicals in order to neutralize them. There is need for a balance between production of ROS and that of anti-oxidant defenses.

Down-regulation of antioxidant defense systems and overproduction of ROS induce oxidative stress, which damages biomolecules, which include proteins, DNA and lipids. The resulting oxidative damage is associated with formation of chronic diseases such as carcinogenesis (Uttara *et al.*, 2009).

Different cancer cells consists of elevated ROS levels compared to non-cancer cells, as a result ROS over-production can be used as a selective tumor suppressor in cancer treatment (Behrend *et al.*, 2003, Hileman *et al.*, 2004, Toyokuni *et al.*, 1995).

1.1.4.1.1. Keap-1/Nrf2 Pathway

Keap-1/Nrf2 pathway is one of the key regulators of the cytoprotective responses to oxidative damage (Kansanen *et al.*, 2013, Zoja *et al.*, 2014). Nrf2 (nuclear factor erythroid 2-related factor 2) is the primary antioxidant response regulatory protein belonging to the Cap'n'Collar subfamily of the bZIP transcription factors (Velichkova and Hasson, 2005, Taguchi *et al.*, 2011, Tufekci *et al.*, 2011). It is an important protein essential for defense system against oxidative stress, thus maintaining the redox balance in the cell (Zoja *et al.*, 2014). Under normal cell conditions Nrf2 is bound to the cystolic repressor protein Keap1 (Kelch ECH associated protein 1) which maintain Nrf2 in the cytoplasm, thus activating its ubiquitin-mediated degradation (Kansanen *et al.*, 2013, Zoja *et al.*, 2014, Nguyen *et al.*, 2009).

Activation of Nrf2 stimulates the anti-inflammatory and antioxidant response and activation of Keap1 represses Nrf2 activation. Upon exposure to oxidative insults, Nrf2 dissociates from Keap1/Nrf2 complex and translocate to the nucleus.

In the nucleus NRF2 forms a heterodimer with small Maf protein, binding to the antioxidant response elements (ARE) at the promoter regions of cellular defense enzyme genes, thereby promoting their transcription (Figure 6) (Nguyen *et al.*, 2009, Zoja *et al.*, 2014, Kansanen *et al.*, 2013, Velichkova and Hasson, 2005). Although Nrf2 is important in cancer chemoprevention in normal tissues, in malignant cells Nrf2 enhances their chemo resistance thereby increasing tumour growth (Sporn and Liby, 2012). Lungs are respiratory organs characterized by elevated Nrf2 levels. This high Nrf2 concentration is what makes lung cells resistant to high levels of ROS produced during respiration. It has been reported that Nrf2 can induce A549 lung cancer cells proliferation (Mitsuishi *et al.*, 2012).

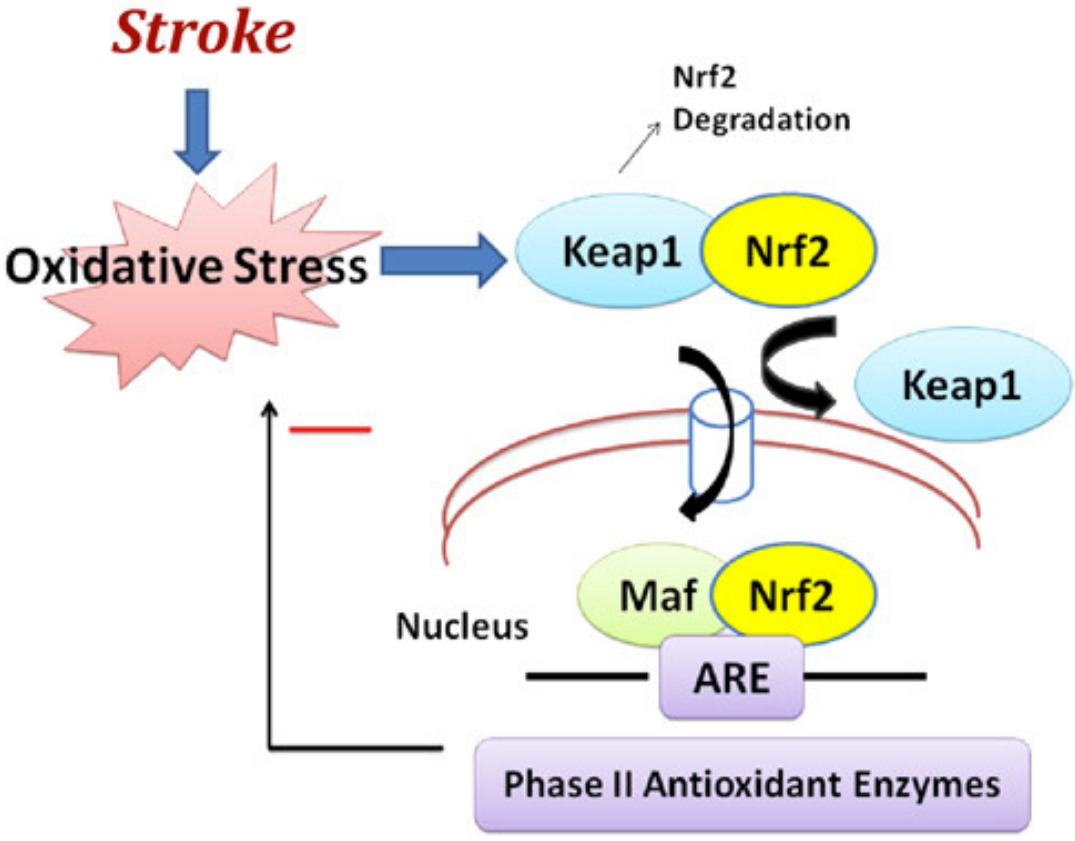


Figure 6: Nrf2/Keap1 signaling pathway (Lakhan *et al.*, 2009).

1.1.4.1.2. Superoxide dismutase (SOD)

SOD are a group of anti-oxidant enzymes that activates superoxide anion (O_2^-) dismutation into a molecular oxygen or hydrogen peroxide. The resulting products are further converted into molecular oxygen and water by catalase thus ensuring that the cellular O_2^- are under physiological conditions (Pelicano *et al.*, 2004). The three different types of SOD are classified according to their protein fold structure and metal cofactor. They include copper-zinc SOD found in mammals, manganese-iron SOD found in mitochondria and bacteria and nickel SOD present in prokaryotes.

Humans and other mammals possess three types of SOD which are localized in different compartments, SOD1 is a copper –zinc SOD found in cytoplasm, SOD2 is a mitochondria localized manganese SOD and SOD3 is an extracellular copper zinc SOD (Alscher *et al.*, 2002, Abreu and Cabelli, 2010).

1.1.4.1.3. Heat shock protein70 (Hsp70)

Hsps are a large family of conserved proteins primarily functioning as molecular chaperones (Mayer and Bukau, 2005, Castelli *et al.*, 2004). Heat shock protein 70 (Hsp70) is a 70-kDa protein essential for the folding of the newly synthesized proteins, protein translocation and refolding of misfolded and/ or aggregated proteins. They are involved in cellular protection against oxidative stress.(Castelli *et al.*, 2004, Mayer and Bukau, 2005, Feng *et al.*, 2006).

Overexpression of Hsp70 is induced by a number of stress stimuli through stress-activated protein kinase (SAPK) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling cascades (Juhasz *et al.*, 2013). Hsps are able to promote cell growth, suppress senescence and inhibit stress induced apoptotic pathways; as a result Hsp70 overexpression has been reported in various cancer cells including NSCLC (Calini *et al.*, 2003, Bonay *et al.*, 1994, Lehman *et al.*, 1991).

1.1.5. Apoptosis

Apoptosis is a programmed cell death employed by the cell or organism for development and as a host defense mechanism (Chang and Yang, 2000).
It is responsible for elimination of damaged cells thus maintaining cellular homeostasis. Cells undergoing apoptosis are characterized by a series of morphological characteristics such as cell shrinkage, plasma membrane blebbing, DNA fragmentation and chromatin condensation (Chang *et al.*, 2013, Wu, 2001, Wong, 2011).

Apoptotic cells can be further characterized by biochemical features which include expression of surface markers such as phosphatidylserine (PS), the recognition ligand functioning as a signal for macrophages to phagocytose dying cells (Elmore, 2007). PS is a negatively charged phospholipid located inside the plasma membrane. Its translocation can be detected using Annexin V protein which has a high affinity for PS (Hingorani *et al.*, 2011).

In order to develop novel and effective anticancer compounds to treat different cancers including lung cancer, understanding the pathways of apoptosis is crucial. This is mainly because most anticancer treatments exert their cytotoxicity to different cancer cells through the apoptotic pathway. Activation of a family of cysteinyl-aspartate proteases (caspases) is one of the main signaling cascades involved in apoptotic pathway (Chang and Yang, 2000).

1.1.5.1. Caspases

Caspases are a group of enzymes involved in pathways essential for maintaining cellular homeostasis based on their regulation of cell death and inflammation. They are synthesized as inactive zymogens or precursors, which are activated through proteolytic cleavage (Beere, 2005, Parrish *et al.*, 2013, Chang and Yang, 2000, McIlwain *et al.*, 2013).

The primary structure of caspases is composed of an amino-terminal pro-domain and a carboxy-terminal protease domain containing the cysteine residue (Parrish *et al.*, 2013). Based on their position in the apoptotic signaling pathway, caspases are grouped into initiator or inducer caspases (caspase-2, -8, -9 and -10) and effector caspases (caspase-3, -6 and -7).

The initiator caspases are characterized by longer amino-terminals and are activated through dimerization with adaptor molecules (Muzio *et al.*, 1998, Chang *et al.*, 2003, Boatright *et al.*, 2003, Pop *et al.*, 2006). Effector or executioner caspases have shorter pro-domains and are responsible for proteolytic degradation of targeted proteins leading to cell death.

Effector caspases activation is executed by proteolytic cleavage between the small and large subunits by initiator caspases (Parrish *et al.*, 2013, Riedl and Shi, 2004).

1.1.5.1.1. Caspases and apoptosis

Apoptosis is initiated through the intrinsic (mitochondrial) and extrinsic (death receptor) pathways based on the exerted signal and caspases activated (Figure 7). The relationship between caspase activity and apoptosis was previously demonstrated on A549 lung cancer cells whereby polyporenic acid inhibited cell proliferation of A549 cells through a caspase-8-mediated apoptosis (Ling *et al.*, 2009).

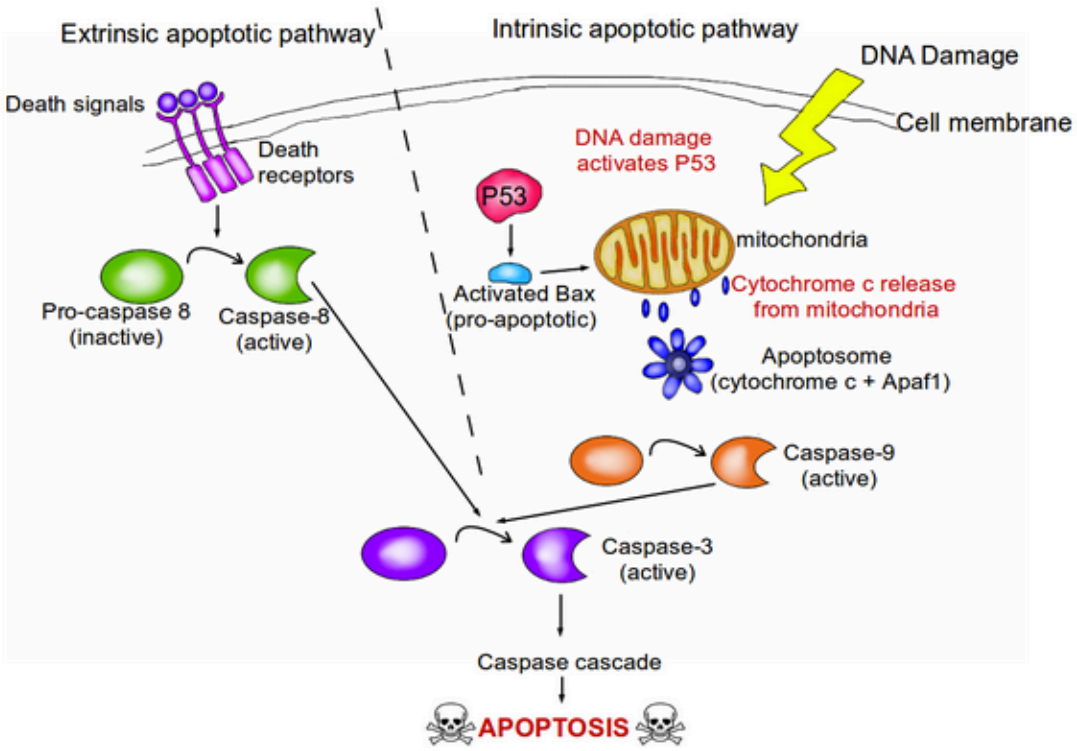


Figure 7: Schematic diagram of apoptosis, showing both extrinsic and intrinsic apoptotic pathways.

(http://blogs.scientificamerican.com/guest-blog/2013/10/02/the-hallmarks-of-cancer-3-evading-apoptosis/)

1.1.5.1.1.1. The extrinsic apoptotic pathway: death receptor pathway

The extrinsic apoptotic pathway is a ligand-induced pathway, which involves activation of death receptors at the plasma membrane in order to remove unwanted cells in growing organisms. The pathway is initiated when an extracellular death ligand bind to a death receptor at the cell surface. Commonly reported death receptors include tumour necrosis factor receptor type 1 (TNFR1) superfamily and its related protein Fas (also known as CD95 or APO-1) (Hengartner, 2001, McIlwain *et al.*, 2013). A typical structure of these death receptors consists of an extracellular region with cysteine-rich domains (CRDs), which are essential for ligand binding and an intracellular region containing a death domain (DD).

Upon ligation of death receptor ligand and death receptor, oligomerization is induced at the cell surface resulting in the formation of death-inducing signaling complex (DISC) (Boatright *et al.*, 2003, Wachmann *et al.*, 2010).
The adaptor proteins including: Fas-associated death domain (FADD) and TNF-related (TNFR) associated death domain (TRADD) are then recruited to the cytoplasmic tail (Algeciras-Schimnich *et al.*, 2002). FADD and TRADD then recruit caspase-8 zymogens by forming a homophilic interaction with their N-terminal death effector domains (DEDs) (Kischkel *et al.*, 1995, McIlwain *et al.*, 2013).

This activates dimerization and activation of caspase-8. Depending on cell type, caspase-8 activation by the DISC may result in subsequent cleavage and activation of downstream caspases (caspase-3 and -7) thus inducing apoptosis. However, for other cells including type II cells mitochondrial induced apoptotic signal is essential for a cell to undergo apoptosis.

1.1.5.1.1.2. The intrinsic apoptotic pathway: mitochondrial-mediated pathway.

This pathway is triggered by a vast array of internal stimuli such as DNA damage, accumulation of unfolded proteins, mitochondrial damage and cytotoxic drugs (McIlwain *et al.*, 2013). Caspase-9 is responsible for the initiation of this pathway. The intrinsic mitochondrial-mediated apoptotic pathway involves mitochondrial polymerization which results in release of cytochrome *c* into the cytoplasm and pro-caspase-9 activation (Wong, 2011). The pathway is tightly regulated by Bcl-2 family of proteins which are classified into anti-apoptotic proteins and pro-apoptotic proteins (Tsujimoto *et al.*, 1984). The pro-apoptotic proteins are responsible for induction of mitochondrial polymerization and the release of cytochrome *c*.

The released cytochrome *c* then interacts with the adaptor protein known as Apaf-1 (apoptotic protease activating factor-1) resulting in the formation of the heptameric backbone of the apoptosome complex (Liu et al., 1996, Zou et al., 1997, Acehan et al., 2002). The apoptosome complex is a seven-spoked wheel consisting of a central hub with a caspase-9 recruitment domain afforded by the caspase-activating recruitment domain (CARD) of Apaf-1.

As a consequence the apoptosome induces conformational change consequently recruiting and activating the initiator caspase-9 through dimerization (McIlwain *et al.*, 2013). The activated caspase-9 subsequently cleaves and activates the downstream effector caspases such as caspase-3 and -7 which execute apoptosis through proteolytic cleavage of proteins such as PARP-1 thereby inducing cell death (Slee *et al.*, 1999).

1.1.6. p53

p53 is a DNA-binding phosphoprotein with a molecular weight of 53 kDa (Bai and Zhu, 2006, Wong, 2011). It was firstly identified around 1978 (Bai and Zhu, 2006, Linzer and Levine, 1979, DeLeo *et al.*, 1979, Lane and Crawford, 1979). Initially p53 was thought to be weakly oncogenic, however later work showed its oncogenic property was found to have resulted from a p53 mutation which was then later called 'guardian of the genome' (Bai and Zhu, 2006, Sigal and Rotter, 2000).

1.1.6.1. The structure of p53

p53 is a tumour suppressor protein normally existing as a homotetramer or as a complex of tetramers (Bellamy, 1997). It is encoded by TP53, the tumour suppressor gene and is a member of a highly conserved protein family consisting of two other members: p63 and p73 (Kaghad *et al.*, 1997, Schmale and Bamberger, 1997). The structure of the wild-type p53 (wt p53) protein consists of 393 amino acids, with an N-terminus consisting of an amino-terminal domain of about 1-42 residues, and a proline-rich region (61-94 residues) (Vousden and Lu, 2002, Slee *et al.*, 2004, Bode and Dong, 2004a).

The C-terminal region (301-393 residues) contains an oligomerization domain (324-355 residues), a nuclear localization signal (NLS) sequence, carboxyl-terminal regulatory domain (363-393 residues) and three nuclear export signal (NES) sequence (Figure 8).

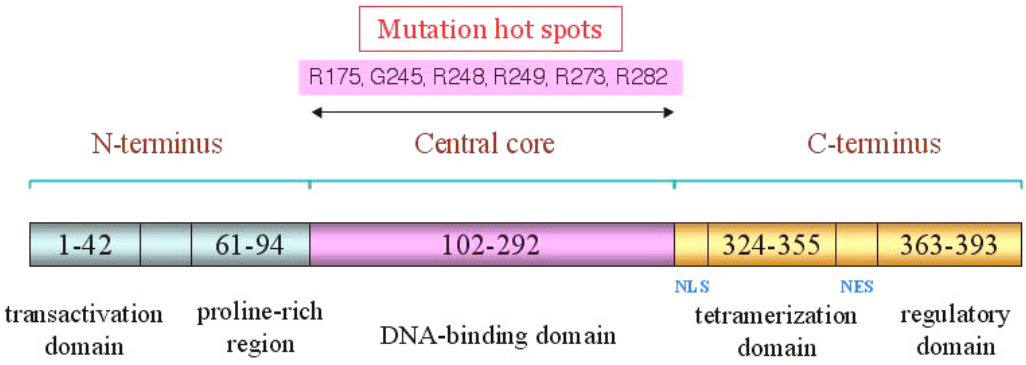


Figure 7: The representation of the p53 structure (Bai and Zhu, 2006).

The amino-terminal region of p53 is essential for transactivation, and it is a point of interaction with transcription factors such as MDM2 (murine double minute 2) or Hdm2 (in humans) (Lin *et al.*, 1994, Fields and Jang, 1990). The proline region is essential for p53 stability which is regulated by MDM2 (Sakamuro *et al.*, 1997).

The central core of p53 contains a consensus sequence known as the DNA binding domain required for the sequence-specific DNA binding (Kern *et al.*, 1991).

1.1.6.2. The physiological functions of p53

p53 is a regulatory protein responsible for regulating expressions of different targeted genes in response to insults present in the cell and the environment.

It is involved in pathways, which control uncontrolled cell proliferation in order to maintain genome integrity after genotoxic stress (Vousden and Lu, 2002, Vogelstein *et al.*, 2000). P53 is a sequence specific transcription factor and a tumour suppressor protein, which is commonly, mutated in human cancers (Beckerman and Prives, 2010, Bellamy, 1997).

The protein DNA binding domain of p53 is responsible for over 80% of cancer-derived p53 mutations (Olivier *et al.*, 2002).

p53 mutation is accounted to by factors including its sensitivity to single base change in the coding sequence and change in phenotype due to loss of allele (Bellamy, 1997). This protein plays an important role in inhibition of tumorigenesis through regulation of cell cycle, differentiation, development, gene amplification, chromosomal segregation, DNA recombination, senescence and induction of apoptosis (Murray-Zmijewski *et al.*, 2008, Vousden and Lu, 2002, Oren and Rotter, 1999).

1.1.6.3. Regulation of p53 levels and activity

Under normal cell conditions Mdm2 maintains wt p53 at low concentrations as well as in an inactive form. Mdm2 is a negative regulator of p53, which interacts with p53 at the N-terminus thus inhibiting its transcriptional activity (Chen *et al.*, 1994, Brooks and Gu, 2003). Furthermore, Mdm3 has been reported to translocate p53 from the nucleus to the cytoplasm for ubiquitin-mediated degradation (Freedman and Levine, 1998).

However, various stimulus such as DNA damage, hypoxia and heat shock result in p53 activation. p53 activation follows three steps: stabilization, sequence specific DNA binding and transcriptional activation of target genes (Figure 9) (Yee and Vousden, 2005).

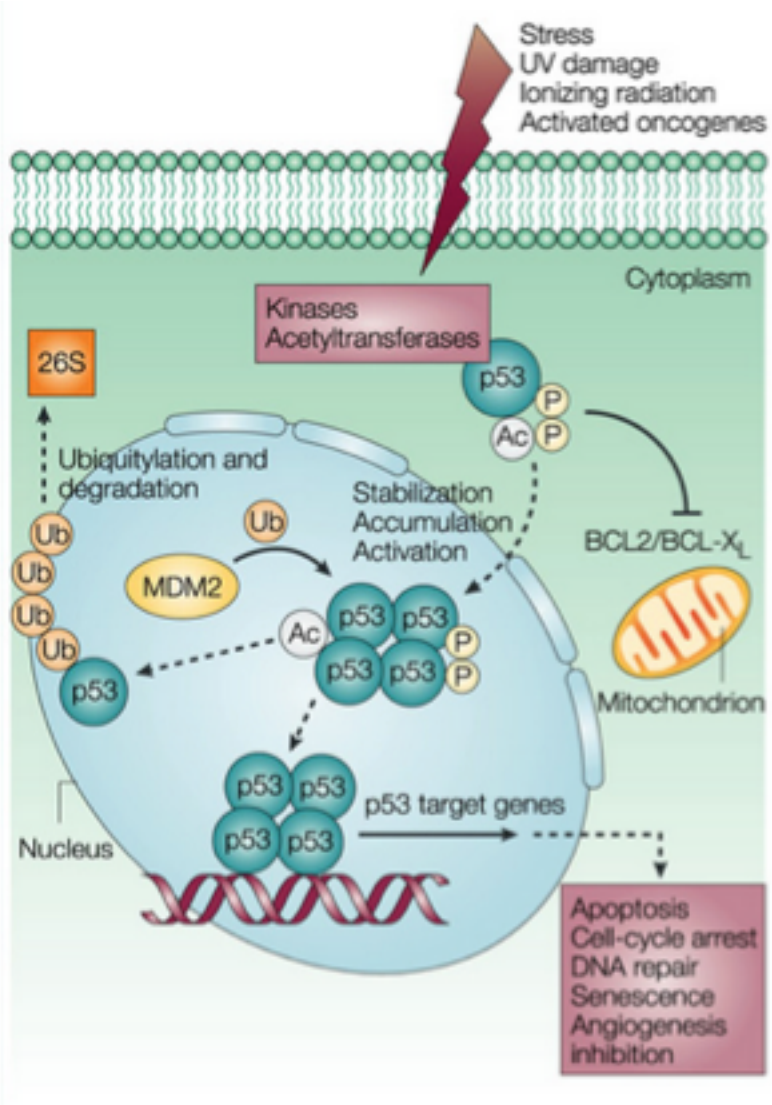


Figure 8: Schematic representation of p53 activation. (Bode and Dong, 2004b).

During p53 stabilization, DNA damage induces post-translational phosphorylation of p53 at the amino terminus thus disrupting the Mdm2-p53 interaction and activating p53.

p53 phosphorylation and acetylation upon genotoxic stress enhance transcriptional activation of p53 resulting in its stabilization and accumulation in the nucleus (Appella and Anderson, 2001, Xu, 2003). Phosphorylation of p53 at Ser46 in tumour cells induces p53-mediated activation of apoptotic-targeted genes (D'Orazi *et al.*, 2002, Bai and Zhu, 2006).

1.1.6.4. p53 and cell cycle

DNA damage induces activation of ataxia-telangiectasia mutated (ATM), which in turn activates checkpoint kinase 2 (Chk2) kinase (Matsuoka *et al.*, 1998). Both ATM and Chk2 consequently result in p53 phosphorylation inducing cell cycle arrest or apoptosis (Banin *et al.*, 1998, Canman *et al.*, 1998). Activated p53 is able to induce cell cycle arrest in the G_1, G_2 and S phases within the cell cycle (Agarwal *et al.*, 1995).

p53 induces cell cycle arrest at G_1 and G_2 phases to provide the cell with an additional time to repair genomic damage prior to entering the important stages of DNA synthesis and mitosis. During cell cycle arrest, the p53 biochemical functions releases the arrested cells back into the proliferation pool, thus allowing for DNA repair processes such as base excision repair and nucleotide excision repair (Zhou *et al.*, 2001). Upon cellular stress, p53 induces G_1 arrest through transcriptional activation of the cyclin-dependent kinase inhibitor, p21 (el-Deiry *et al.*, 1993, Chen *et al.*, 1996).

1.1.6.5. p53 and apoptosis

In the case whereby DNA damage is too severe to be repaired, p53 induces apoptosis of cell with damaged DNA. A p53-dependent apoptosis was first reported in 1993 after irradiation of mouse thymocytes by Clarke and co-workers and also by Lowe and co-workers (Zilfou and Lowe, 2009, Clarke et al., 1993). The ability of p53 to regulate cell cycle and to induce of apoptosis has resulted in the increased enthusiasm to study its properties as a potential target for anticancer therapy. p53-mediated apoptosis is based on p-53 localization to the mitochondria where it modulate the expression of targeted proteins such as the Bcl-2 family of proteins which control mitochondrial permeability (Bai and Zhu, 2006).

1.1.1.6. Bcl-2 family of apoptosis and apoptosis

Bcl-2 family of proteins consists of proteins with both cell-damage and cell-survival signals. Bcl-2 was the first protein to be identified in this family and it obtained its name from B-cell lymphoma/leukaemia-2, the human B-cell proteins capable of chromosomal translocation (Kroemer *et al.*, 2009, Vaux and Silke, 2003, Tsujimoto *et al.*, 1984). Bcl-2 proteins are dimers located on the outer mitochondrial membrane and they are responsible for induction of membrane permeability though creation of outer membrane pores.

Over 25 members of Bcl-2 proteins have been identified and based on their function and Bcl-2 homology (BH) they are broadly divided into three groups: the anti-apoptotic proteins (Bcl-2 and Bcl-X_L) which contain four BH domains, pro-apoptotic proteins (Bax, Bak and Bcl-XI) also containing four BH domains and pro-apoptotic "BH3-only" proteins (Bid, Bad, Noxa and Puma) (Haupt *et al.*, 2003). In addition to the BH domains, Bcl-2 protein family also consists of a carboxy-terminal hydrophobic domain responsible for membrane localization (Adams and Cory, 1998, Green and Reed, 1998).

Bcl-2 is a 25-26 kDa protein residing in the cytoplasmic face of mitochondrial outer membrane and its primarily function is to protect the mitochondrial membrane integrity (Miyashita and Reed, 1995, Petros *et al.*, 2004). Bax is a Bcl-2 homolog of 21 kDa and it is mainly localized in the cytosol (Miyashita and Reed, 1995, Petros *et al.*, 2004). During cellular stress such as DNA damage, p53 is activated thereafter activating and inducing Bax localization to the mitochondrial membrane resulting in increased mitochondrial membrane permeability. This results in cytochrome *c* release and the subsequent induction of apoptosis through activation of down-stream apoptotic target proteins.

1.1.7. Carbazoles

For over a century, heterocycles have remained an important class of organic compounds forming a classical division of organic chemistry research.

Their structural flexibility has resulted in their increased use in different fields including medicine, agriculture and industry (Dua *et al.*, 2011, Liu and Larock, 2007). Among heterocycles, carbazoles alkaloids are a group of natural compounds with a growing research interest.

The first carbazole was isolated in 1872 by Graebe and Glazer from coal tar. Carbazoles have now been isolated from plant species including *Murraya Clausena*, *Micromelum* and *Glycosmis* of the Rutaceae family (Majumdar, 2011). Based on their reported pharmaceutical and industrial properties, pronounced effort has been dedicated to develop methods for the effective synthesis of carbazole compounds and their derivatives.

Classical methods used for carbazole synthesis include the Graebe-Ullman and the Fischer-Borsche processes (Majumdar, 2011, Liu and Larock, 2007). Other synthesis techniques include the palladium catalysed procedure and the iron-mediated synthesis.

1.1.7.1. The carbazole structure

Carbazoles and their derivatives are a group of nitrogen containing heterocyclic compounds (Nandy *et al.*, 2014). They have a tricyclic structure with two benzene rings, each attached on either side of a five-structured nitrogen containing ring (Figure 10) (Nandy *et al.*, 2014).

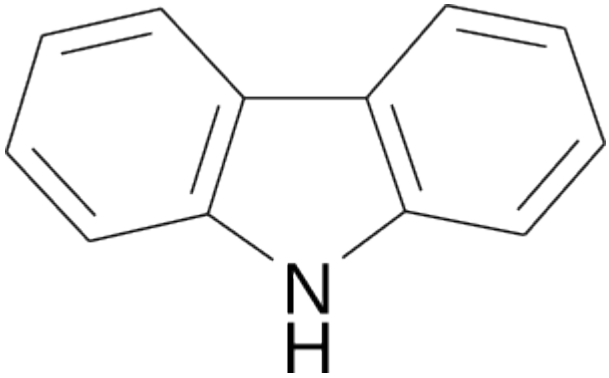


Figure 9: Carbazole structure
(http://www.scbt.com/datasheet-214662-carbazole.html)

The carbazole structure is reported to be dependent on the indole structure whereby the second ring is attached to the five-membered ring of the indole at the 2-3 positions. The increased interest on carbazoles and its derivatives is due to its charge and electronic transport properties, and a large π-conjugated system which makes it easy to introduce different functional groups into a rigid carbazole ring during synthesis (Nandy *et al.*, 2014).

1.1.7.2. Biological properties of carbazoles

Carbazoles and their derivatives have been reported to have a spectrum of pharmaceutical properties, which include: antimicrobial, anti-HIV, anti-arthritis, anti-diabetic and anticancer activities(Kongkathip *et al.*, 2005, Kaplancikli *et al.*, 2012, Roy *et al.*, 2005). The anticancer activity of carbazoles and its derivatives has been associated with the ability of their structures to intercalate into the DNA thus inhibiting the activity of DNA topoisomerase II. Their anticancer activities can further be attributed to the formation of covalent DNA adducts which are mediated by their oxidation with cytochromes P_{450} and peroxides (Nandy *et al.*, 2014).

Different natural and synthetic carbazole derivatives including elipticine, olivacine, elliptinium acetate, mahanimbine, mukonine, koenoline and rebaccamycin have been reported to have antineoplastic activity (Nandy *et al.*, 2014).

1.1.8 A549 cells

Potential anticancer pharmaceuticals are studied *in vitro* prior to clinical trials using cancer cell models such A549 cell line. A549 cells are squamous, adenocarcinomic human epithelial cells developed in 1972 from a cancerous lung tissue of a 58-year-old Caucasian male (Praveen and Manoj, 2014). A549 cells are characterized by elevated glutathione levels and high HO-1 gene expression which is involved in their response to oxidative stress (Dubrovskaya and Wetterhahn, 1998). A549 cells also have characteristic features of type II alveolar cells, allowing A549 cells not only to be used as a model for lung cancer, but also as an *in vitro* model to study human primary alveolar epithelial cells (Tian et al., 2009b, Tian et al., 2009a, Dubrovskaya and Wetterhahn, 1998). These cells are also commonly used to study the toxic and genotoxic effects of environmental pollutants, since lungs are the primary biological targets for inhaled toxins (Dubrovskaya and Wetterhahn, 1998, Lee *et al.*, 1996). If cultured, A549 cells grow as adherent or monolayer cells attaching to the flask.

CHAPTER TWO

2.1. MATERIALS AND METHODS

2.1.1. Materials

Carbazole derivative (ECAP) was synthesized at the Department of Chemistry (Durban University of Technology, SA). A549 cells were obtained from Highveld Biologicals (Johannesburg, SA).

Reagents and equipment

Reagents and equipment	**Supplier and product number**
BD Falcon™ Polystyrene round bottomed tubes, 5 ml, 12x75 mm style	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA#352054
FACS flow	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
FACS clean	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
FACS rinse	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
Trypsin EDTA (0.25% 1X)	BioWest, France L0931-100
Trypan blue 0.4%	Sigma-Aldrich, South Africa #T8154
Ethanol	MERCK #SAAR2233540LP
Foetal calf serum, endotoxin free	Delta Bioproducts, Highveld Biological, Lyndhurst, South Africa #14-501-BI
Tween 20	MERCK #6164500KF
acetylacetone	Sigma # P7754
indium chloride	Sigma # 334065
ethyl acetate	MERCK # SAAR2235020LC
anhydrous sodium sulphate	Sigma # 239313
methanol	MERCK SAAR416080LC
Eagle's minimum essential medium	Whitehead Scientific (Johannesburg, SA)
1% L-glutamine	Whitehead Scientific (Johannesburg, SA)
1% penicillin-streptomycin-fungizone	Whitehead Scientific (Johannesburg, SA)
A549 lung cancer cells	HIGH VELD
DMSO	MERCK #K42708912 147
Spectrophotometer	Bio Tek µQuant
gel red	Sigma # S5817
LMP agarose	Life technologies # 16520
NaCl	MERCK #SAAR5822320EM

Triton X-100	MERCK # 9036-19-5 OR
EDTA	Sigma-Aldrich, South Africa # E9884
Fluorescence microscope	Olympus IXSI
Cytobuster reagent supplemented with phosphate inhibitor	Roche, cat. no. 04906837001
protease inhibitor	Roche, cat. no. 05892791001
Bicinchoninic acid assay	Sigma, Germany
p53	Cell Signaling Technology # 2521P
Nrf2	Cell Signaling Technology # 8882
SOD	Cell Signaling Technology # 4266
Hsp70	BD Transduction laboratories # BD 610607
1 mM Tris	Sigma
Bcl-2	Cell Signaling Technology # 3869
anti-mouse	Abcam # ab97046
β-actin	Abcam # ab8226
Clarity Western luminal/enhancer solution	BIO-RAD
Clarity Western peroxide solution	BIO-RAD
Alliance 2.7 image documentation system	UViTech
caspase-Glo 3/7 reagent	Promega
caspase-Glo 8 reagent	Promega
caspase-Glo 9 reagent	Promega
ATP reagents	Promega
Annexin-V-FLUOS Staining Kit	Roche # 11 858 777 001
BD Accuri™ C6 flow cytometer	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
MDA	Sigma-Aldrich, South Africa # 10838-3
2-Mercaptoethanol	MERCK #4161200
APS	Sigma-Aldrich, South Africa # A3678
Tris	MERCK 77086-1 /108382-0500
SDS	BDH #301754L
Glycine	MERCK SAAR2676600EM
copper sulphate solution	Sigma-Aldrich, South Africa # C2284
Bis-acrylamide	Sigma-Aldrich, South Africa # A3449
TEMED	MERCK #1.10732.0100
Thiazoylblue tetrazolium bromide	Sigma-Aldrich, South Africa # M5655
BSA	ROCHE #10735086001
Cytobuster reagent supplemented with phosphate inhibitor	Roche # 04906837001
protease inhibitor	Roche # 05892791001

2.1.2. Synthesis of (Z)-4-((9-ethyl-9H-carbazol-3-yl)amino)pent-3-en-2-one (ECAP)

A mixture of acetylacetone (1.02 mL, 0.01 mol), 3-amino-9-ethylcarbazole (2.1 g, 0.01 mol) and indium chloride (0.22 g, 0.001 mol) in ethanol (25 mL) was heated under reflux for 5 h (Figure 11). After the reaction was completed, the excess of solvent was evaporated. The resulting residue was then dissolved in ice/water and extracted with ethyl acetate. Anhydrous sodium sulphate was used for drying the combined organic layers. Silica gel column (eluent–petroleum ether: ethyl acetate (90:10) was then used for purification. The pure product was recrystallized from methanol.

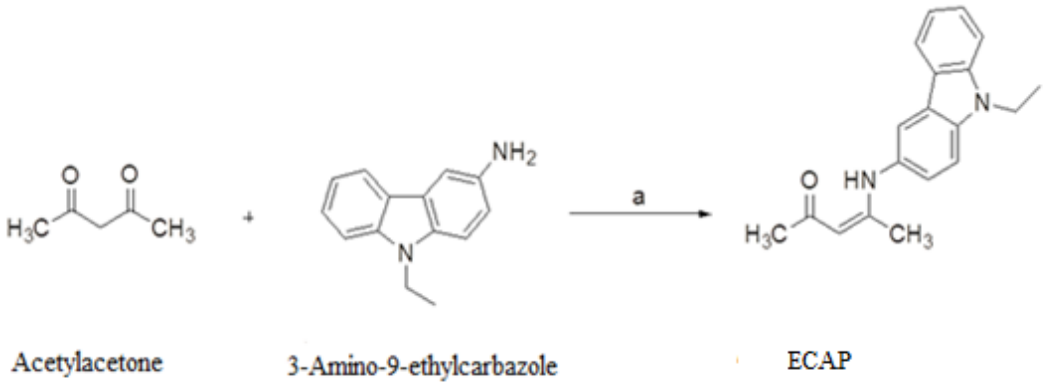


Figure 10: The schematic representation of the ECAP:$InCl_3$/Ethanol, reflux, 5h RT.

The title compound (Z)-4-((9-ethyl-9H-carbazol-3-yl)amino)pent-3-en-2-one (ECAP) was prepared in good yield by two component reaction under indium chloride as promoter structure of the prepared compound. The structure of the prepared compound was then studied and characterized using Infrared (IR), Hydrogen-1 (^{1}H) and Carbon-13 (^{13}C NMR) spectroscopic techniques.

2.1.3. Tissue cell culture

The A549 lung cancer cells were cultured in 25 cm^3 culture flasks at 37ºC, 5% CO_2 in complete culture media (CCM) which consisted of Eagle's minimum essential medium (EMEM) supplemented with 1% L-glutamine, 1% penicillin-streptomycin-fungizone and 10% foetal calf serum. Cell growth was monitored and cell culture medium (CCM) was changed as required. Confluent flasks were trypsinized and trypan blue was used for cell numeration.

2.1.4. Cell viability assay

2.1.4.1. MTT assay

2.1.4.1.1. Theory

The methyl thiazol tetrazolium (MTT) assay was used in order to determine the cytotoxicity of ECAP on A549 lung cancer cells. This is a colorimetric cell viability assay based on the reduction of the yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt into a purple formazan in the mitochondria of viable cells by mitochondrial succinate dehydrogenase, Figure 12 (Stockert *et al.*, 2012, Bopp and Lettieri, 2008). The resulting formazan is then solubilized with organic solvent (e.g. propan-2-ol (iso-propanol archaic)) and measured spectrophotometrically.

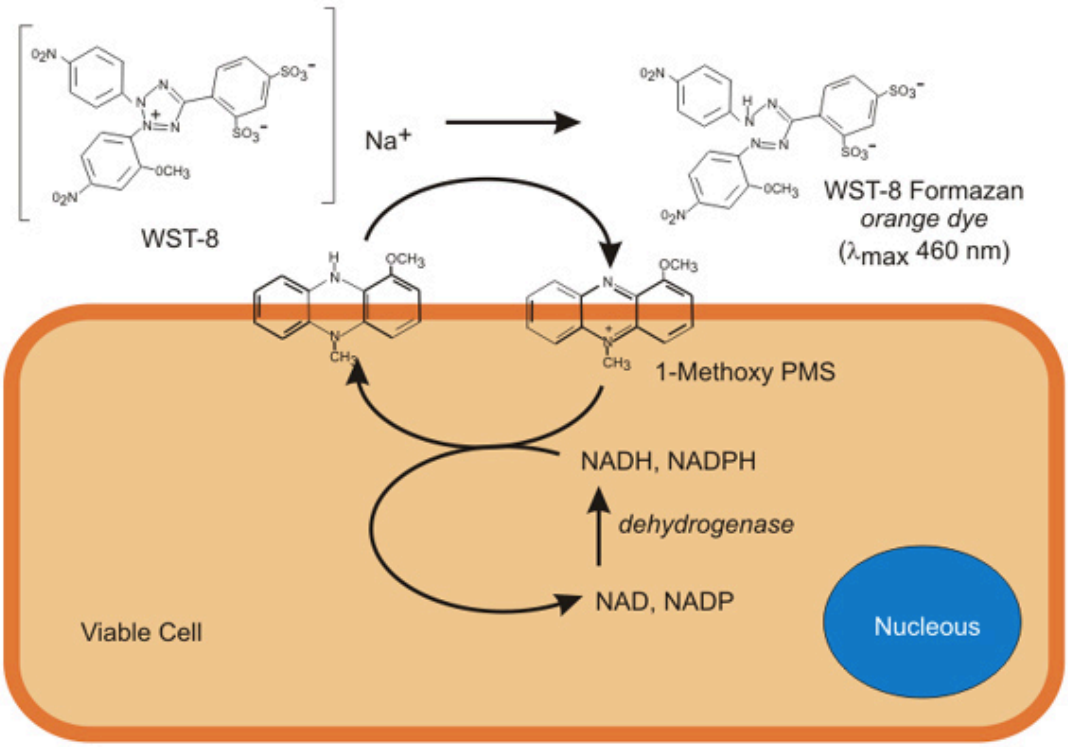


Figure 11: A schematic representation of cytotoxic assays (Held, 2009).

2.1.4.1.2. Method

A549 cells (20, 000 cells/well) were treated in triplicate with different concentrations of ECAP (0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.6, 0.7 and 0.8 µg/ml) and incubated at 37ºC, 5% CO_2 for 24 h in 96-well microtitre plate. Cells incubated with CCM/DMSO (dimethyl sulphoxide) were used as vehicle control (V control). CCM/MMT solution was prepared by mixing 20 µl MTT (5 mg/ml in 0.1 M PBS) and 100 µl fresh CCM (per well). After 24 h incubation, the media was removed and cells were washed three times with 0.1M phosphate buffer saline (PBS) and then CCM/MTT salt solution was added into each well and incubated at 37ºC for 4 h. The supernatant was removed and 100 µl/well DMSO was added followed by 1 h incubation at 37ºC.

The absorbance of the produced formazan was measured at 570 nm and reference wavelength of 690 nm using a Bio Tek µQuant spectrophotometer. GraphPad Prism V5.0 software was used to plot a concentration-response curve that was subsequently used to determine an IC_{50} value of ECAP on A549 cells.

2.1.5. Lipid peroxidation assay for quantification of malondialdehyde (MDA)

2.1.5.1. Thiobarbituric acid assay (TBARS)

2.1.5.1.1. Theory

Lipid peroxidation is a chain reaction characterized by hydrogen abstraction or oxygen radical addition thus inducing oxidative damage of polyunsaturated fatty acids (PUFA) (Repetto *et al.*, 2012). Different byproducts of lipid peroxidation including malondialdehyde (MDA) are produced as a result of oxidation of PUFAs (Figure 13) (Hodges *et al.*, 1999). The produced MDA is known to react with thiobarbituric acid (TBA) in an acid-catalyzed nucleophilic reaction producing a pinkish-red chromogen with an absorbance of 532 nm. Therefore, thiobarbituric acid assay (TBARS) was used to determine the ability of ECAP to induce lipid peroxidation.

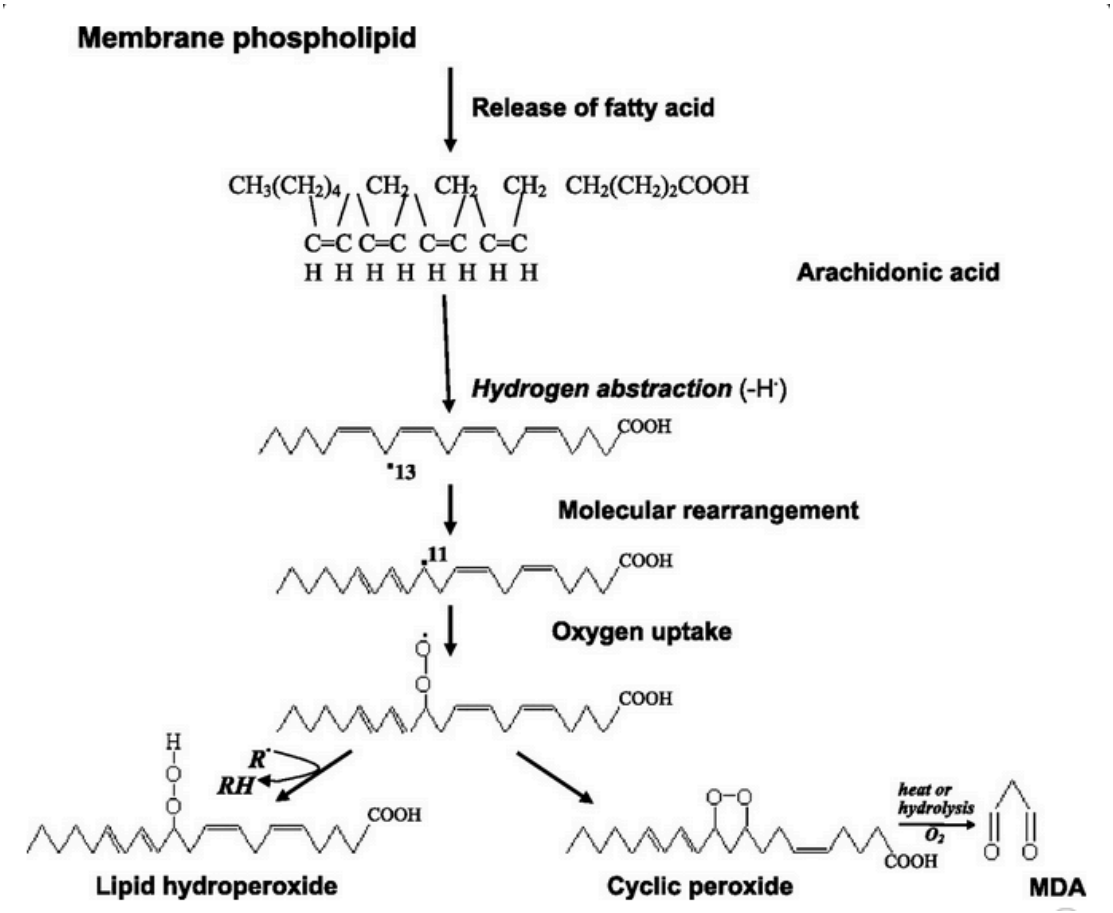


Figure 12: Stages of lipid peroxidation (Powers and Jackson, 2008)

2.1.5.1.2. Method

A549 cell were treated for 24 hr. Thereafter the supernatant of the control, V control and ECAP treated cells was transferred into test tubes with 2% H_3PO_4 (200 µl), TBA/BHT solution (400 µl) and 7% H_3PO_4. A positive control of 1% MDA and a negative control of 3mM HCl (400 µl) was used. Samples pH was checked (pH 1.5) and heated at 100ºC for 15 min. Samples were allowed to cool down at room temperature (RT), followed by addition of 1.5 ml butanol, vortexed and then allowed to separate into distinct phases. The upper butanol phase was aliquoted into 96-well microtitre plate in triplicates.

The spectrophotometer was used to measure the optical at 532 nm with reference wavelength of 600 nm. The mean of the optical density was calculated for each sample and divided by the absorption coefficient of 156 mM^{-1} in order to obtain MDA concentration (μM) per treatment.

2.1.6. DNA damage

2.1.6.1. Comet assay

2.1.6.1.1. Theory

Comet assay or single cell gel electrophoresis (SGE) is fluorescent microscopy-based technique used for detection of DNA damage. Comet assay was originally developed in 1984 by Ostling and Johansson and then modified by Singh and coworkers in 1988 (Olive and Banáth, 2006). Comet is used to describe the pattern of DNA migration during electrophoresis under high pH conditions.

During comet assay cells are encapsulated with a low-melting point agarose suspension, lysed under alkaline conditions followed by electrophoresis of lysed cells (Enciso et al., 2009, Olive and Banáth, 2006). The comet assay is based on the principle that DNA strands containing breaks lose their supercoiling capability and migrate towards the anode forming the comet head (Figure 14). Therefore, cells containing high levels of damaged DNA will have more intense comet tails.

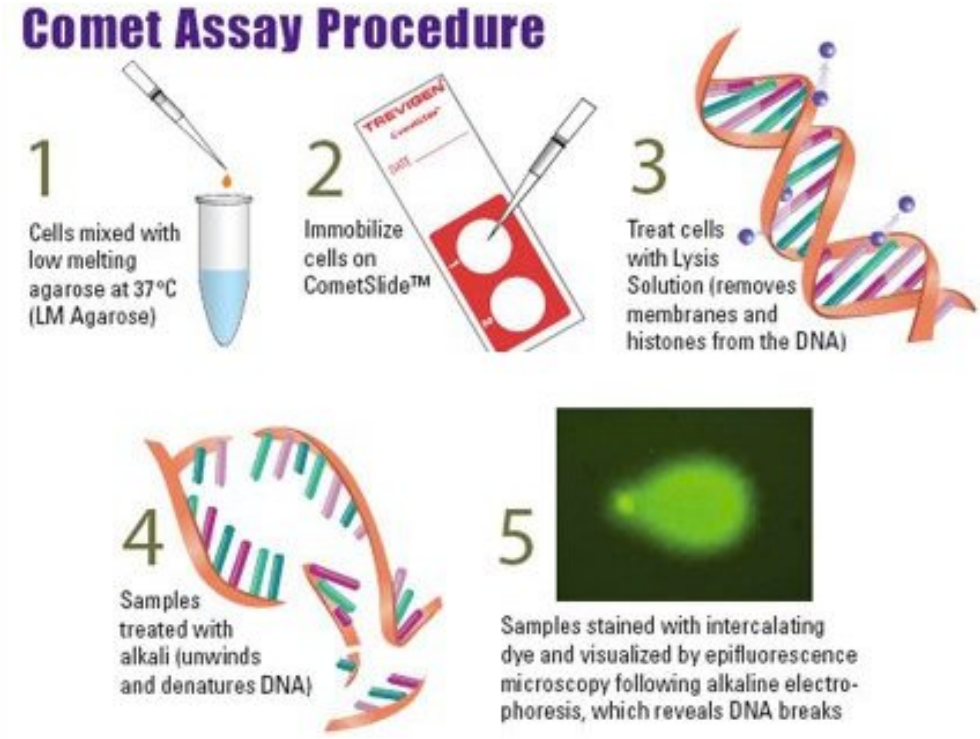


Figure 13: Comet assay. (http://www.amsbio.com/Comet-Assays.aspx)

2.1.6.1.2. Methods

The comet assay was used as previously described by (Tiloke *et al.*, 2013), with slight modification to determine DNA damage. The 24 h treatments (control, V control and ECAP) were prepared respectively followed by removal of supernatant, and then trypsinization. About 20 000 cells/treatment were dissolved in 1 ml 0.1 M PBS. Two microscope slides (76 × 26 mm) per sample were prepared. The first layer of 700 µl, 2% low melting point agarose (LMPA, 37ºC) was added towards the frosted end of the microscope slide, the second layer of cells (25µl), 1% LMPA (175 µl, 37ºC) and gel red (1.5 µl). The third layer of 1% LMPA (250 µl, 37ºC), and then covered with microscope cover slides.

After adding each layer of the gel, a coverslip (60 × 20 mm) was placed on top of the gel and incubated at 4°C for 10 minutes to allow the gel to solidify. After solidification, coverslips were removed and the slides were immersed into cold lysing solution [2.5 M NaCl, 1% Triton X-100, 1 M Tris, 100 mM EDTA, 10% DMSO] and incubated at 4ºC for 1 h. Following lysis, the slides were placed into electrophoresis buffer [1 mM Na_2EDTA (pH 13), 300 mM NaOH] for 20 min, and then electrophoresis was run at 25 V for 35 min.

After electrophoresis, prepared slides were then washed three times for 5 min with neutralizing buffer [0.4 M Tris (pH 7.4)]. Slides were then covered with coverslips and viewed with a fluorescent microscope (Olympus IXSI inverted microscope with 590 nm emission filters and 510-560 nm excitation). For each slide, 50 cells were captured and their comet tail lengths were measured in μm using Soft imaging system (Life Science-Olympus Soft Imaging Solution v5).

2.1.7. Western blot analysis

2.1.7.1. Theory

Western blot is an electrophoresis technique used for protein separation and identification on the basis of molecular weight (Yang and Ma, 2009). Separated proteins are then transblotted onto a membrane, and the membrane is probed with antibodies specific for the protein of interest. Western blot was used to quantify the protein expression of p53, Bax, Bcl-2, Nrf2, SOD and Hsp70 in A549 cells.

2.1.7.2. Method

2.1.7.2.1. Sample preparation

Proteins were isolated from A549 cells using Cytobuster reagent supplemented with phosphate inhibitor and protease inhibitor. Bicinchoninic acid (BCA) assay was used for protein quantification. Proteins were standardized to a concentration of 3.966 mg/ml. Standardized proteins were then prepared in a 5X Laemmli buffer [0.5M Tris-HCl (pH 6.8), dH_2O, glycerol, β-mercaptoethanol, 10% SDS, 1% bromophenol blue] (1:4 ratio) and boiled for 5 min at 100ºC. Sodium dodecyl sulphate (SDS) is a detergent used for protein denaturation and for creating an overall negative charge on the protein to allow protein migration through the gel. β-mercaptoethanol is also involved in protein denaturation based on its ability to reduce disulfide bonds of proteins while glycerol is used to add density to the sample to aid the sample to sink to the bottom of the well when loading into the gel.

2.1.7.2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The prepared sample was allowed to cool at RT before loading into the gel. A 4% stacking gel and 7.5% resolving gels (dH_2O, Tris, SDS, Bis/Acrylamide, 10% Ammonium phosphate sulphide, TEMED (Tetramethylethylenediamine)) were prepared. TEMED was added to initiate N,N'-methylene-bis-acrylamide mediated polymerization of acrylamide gels. After polymerization, molecular weight and samples were added into the gel in duplicates. The gels were then electrophoresed for 1 h at 150 V in 7.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad compact power supply.

2.1.7.2.3. Transfer

Following electrophoresis gels were equilibrated in a transfer buffer consisting of: dH_2O, Tris, glycine and methanol. Equilibrated gels, membranes and fibre pads were prepared into a sandwich between the two electrodes (Figure 15). Trans-Blot Turbo Transfer system was then used to transfer the separated proteins into nitrocellulose membrane at 20 V for 45 min using the BioRad TransBlot Turbo Transfer System (400mA).

After transfer, nitrocellulose membranes were blocked with 3% BSA for 1 h in Tris-buffered saline [(TTBS)-NaCl, Tris, KCl, dH_2O, Tween 20, pH 7.4)] containing 0.5% Tween20.

2.1.7.2.3.1. Transfer method

After the blocking step, membranes were incubated overnight with primary antibodies [p53, Nrf2, SOD, Hsp70 and Bcl-2; 1:5,000 in 1% BSA] followed by a 5X wash with TTBS. Nitrocellulose membranes were incubated with a horseradish peroxide (HRP) conjugated secondary antibodies (RT, 1 h) followed by a 5X wash with TTBS (10 min each).

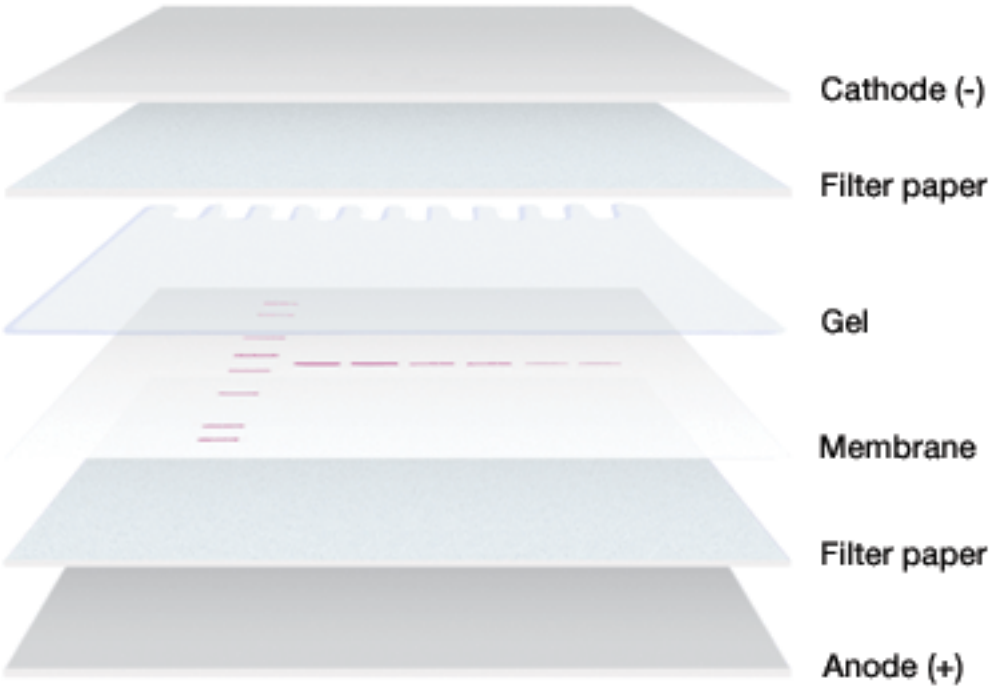


Figure 14: Gel and membrane arrangement for protein transfer onto nitrocellulose membrane.

(http://www.bio-rad.com/en-al/applications-technologies/protein-blotting-methods)

2.1.7.2.4. Detection

A 50:50 (v/v) of Clarity Western luminal/enhancer solution and peroxide solution was added onto each electro-blotted nitrocellulose membrane to form the antigen-antibody complex (Figure 16). The generated signal was detected using the Alliance 2.7 image documentation system (UViTech). Protein expression was then analysed using UViBand Advanced Image Analysis software v12.14 (UViTech). The obtained data was expressed as fold change (FC).

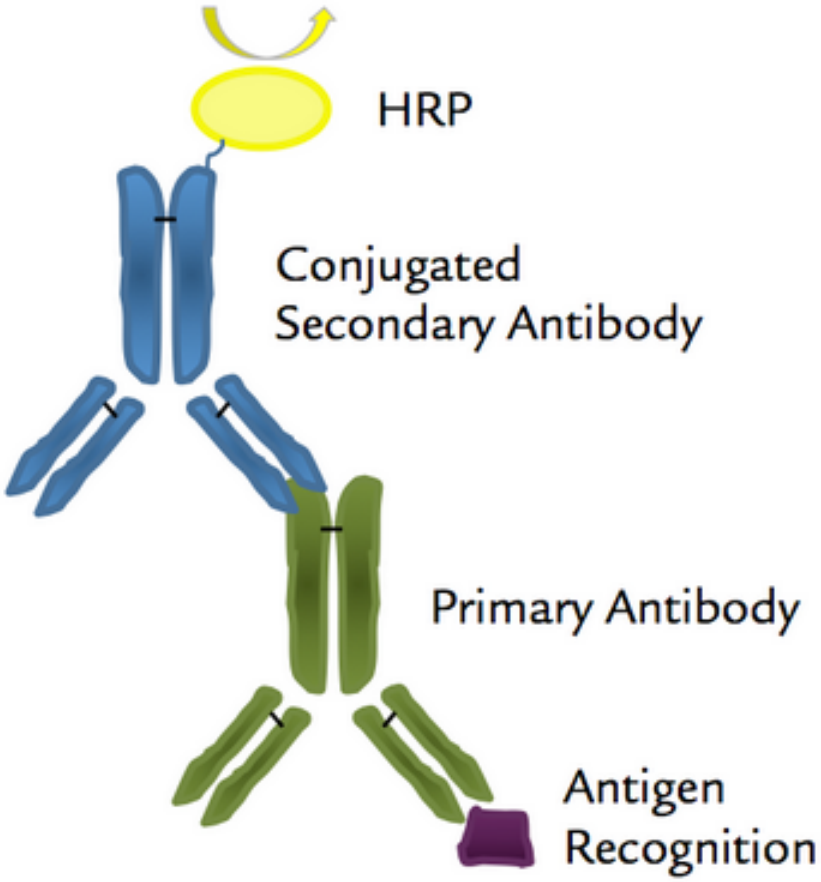


Figure 15: Antibody-antigen reaction for the chemiluminescence detection. (http://www.rockland-inc.com/custom-antibody-conjugation.aspx)

2.1.7.2.4. Normalization

β-actin (ab8226; 1:5,000) was used for protein normalization. After viewing the probed proteins of interest, membranes were stripped with 5% hydrogen peroxide (30 min, 37ºC), washed 1X with TTBS and incubated overnight with β-actin (3% BSA, RT).

2.1.8. Caspase-3/7, 8, 9 and ATP activities

2.1.8.1. Luminometry

2.1.8.1.1. Theory

Luminescence-based assays were used to assess the activities of caspase-Glo 3/7, 8, 9 and ATP. Luminometry is a highly sensitive analytical technique commonly used for measuring chemi- and bioluminescent reactions. ATP can be extracted from cells assayed with luciferin-luciferase luminometry to determine ATP measurements, whereby ATP is a limiting reagent. During ATP analysis, the emitted light is proportional to the amount ATP present in the sample (Figure 17). Luminometry can also be used to determine the caspase activity. This is based on the addition of the caspase reagent that lyses cells and thus activating caspase cleavage of the substrate, generating a luminescent signal. The generated luminescent signal is proportional to the caspase activity.

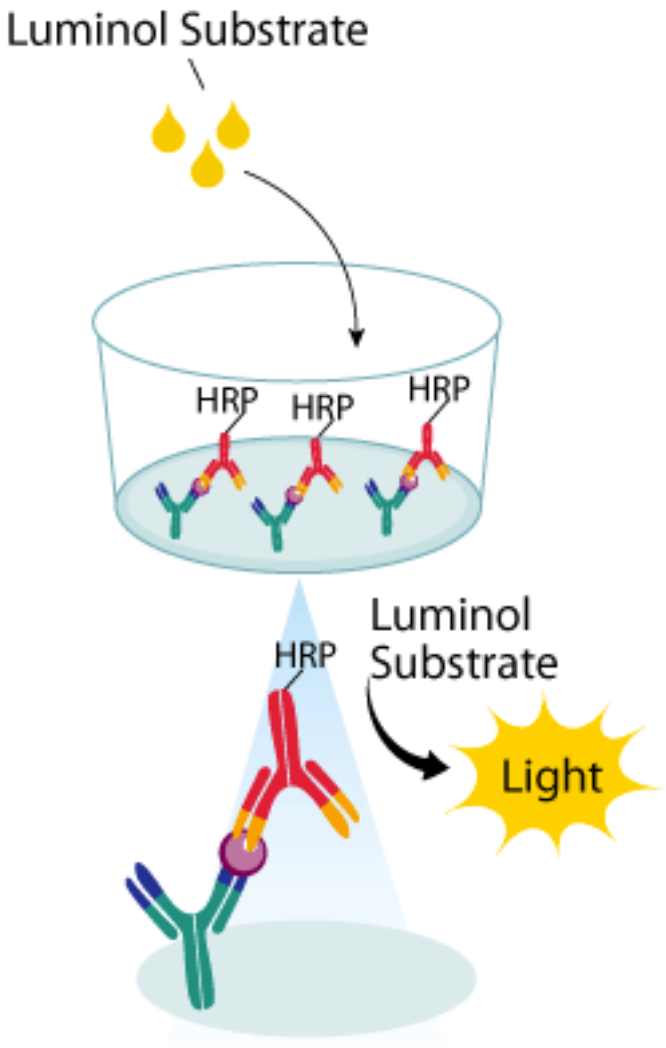


Figure 16: Detection of caspase activity

(http://www.rndsystems.com/product_detail_objectname_quantigloelisaassay

2.1.8.1.2. Method

Following a 24 h treatment with ECAP, A549 cells were trypsinized, adjusted to 20 000 cells/well, followed by centrifugation at 3000g x (5 min, RT). Supernatant was removed; the cells were then re-suspended in 50 µl PBS/well for each treatment and then seeded into an opaque polystyrene 96-well microtitre plate in six replicates.

The manufacture's guidelines were used for preparing Caspase-Glo 3/7, 8, 9 and ATP reagents. About 100 µl of reagent was added into specific wells, and then incubated in the dark (30 min, RT). The Modulus microplate luminometer was used for the subsequent measurement of the luminescence and the obtained data was expressed as RLU.

2.1.9. Annexin-V-Fluos assay

2.1.9.1. Theory

Phosphatidylserine (PS) externalization is a primary characterization of the early stage of apoptosis. PS translocation was determined using annexin-V-Fluos apoptosis detection kit. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein with high affinity for PS (Hingorani *et al.*, 2011). Apoptosis can be detected by flow cytometry using fluorescein isothiocyanate (FITC) labeled Annexin V, whereby this fluorescently labeled Annexin V specifically binds to the exposed phophatidyl-serine on the surface of apoptotic cells (Figure 18) (Hammill *et al.*, 1999, Bossy-wetzel and Green, 2000).

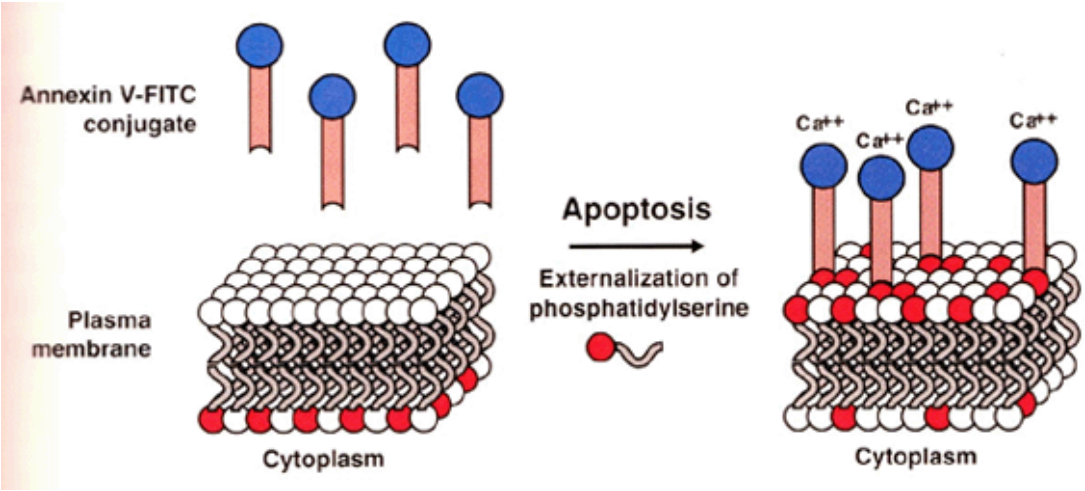


Figure 17: The principle basis of the **A**nnexin V-FITC binding assay (Zhang *et al.*, 1997).

2.1.9.1.2. Method

A549 cells were treated with ECAP for 24 h, tripsinized and centrifuged for 3 min at 2000 xg. The resulting pellet was re-suspended in 200 µl 0.1 M PBS. To each 100 µl A549 cell suspension, 100 µl of Annexin staining buffer and 100µl of Annexin-V-Fluos labelling solution (1 ml Annexin staining buffer + 20 µl annexin-V + 20 µl propidium iodide) were added in 1.5 ml Eppendorfs. The BD Accuri™ C6 flow cytometer and software was used for analysis and capturing of data elucidated by stained cells. Cells were gated using FlowJo v7.1 software (Tree Star Inc., Ashland, USA). About 50 000 events were analysed in triplicate. The obtained results were expressed as percentage of apoptotic cells.

2.1.10. Statistical analysis

GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, USA) was used for statistical analysis. For all experiments, the obtained results of the triplicates were represented as means with standard deviation (SD).

The statistical significance of the results was analysed with unpaired *t*-test and a 95% confidence interval. The values of $p < 0.05$ were then considered statistically significant.

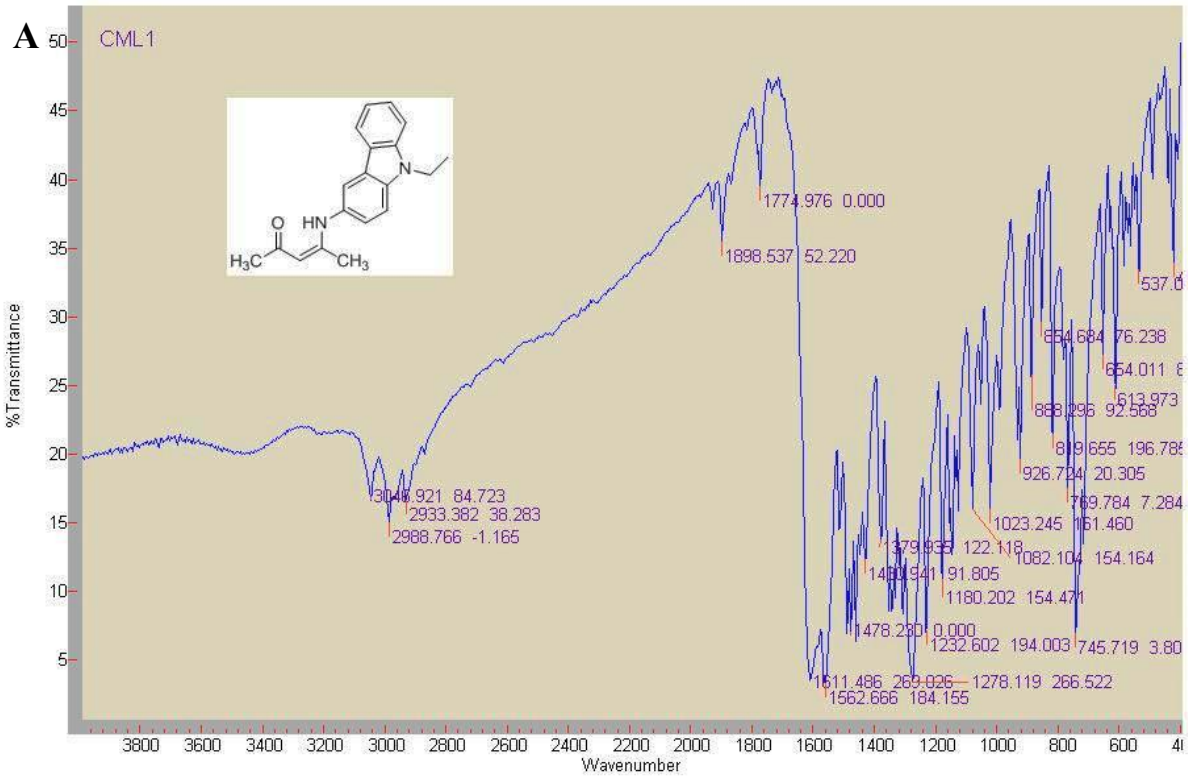
CHAPTER THREE

3.1. RESULTS

3.1.1. Synthesis of (Z)-4-((9-ethyl-9H-carbazol-3-yl)amino)pent-3-en-2-one (ECAP)

The resulting product was a yellow solid produced in 94 % yield; melting point (mp): 240-247 °C. Functional groups were predicted using IR (KBr, cm^{-1}): 1774.97 (C=O), 3043.92 (N-H), 2988.76 (C-H) Alkanes, 1562.66 (C=C) Aromatic rings (Figure 19.A). The ^{1}H-NMR (400 MHz, $CDCl_3$) was used to predict the ratio of the hydrogen number: δ (ppm) 12.59 (s, 1H), 8.20 (q, 1H), 7.8 (d, 1H), 7.45–7.40 (m, 2H), 7.35–7.30 (m, 3H), 5.3 (s, 1H), 4.35 (q, 2H), 2.25 (s, 3H) 1.95 (s, 3H), 1.45 (t, 3H) (Figure 19.B). The ^{13}C-NMR (400 MHz, $CDCl_3$) was further used to predict the backbone of the molecule: δ (ppm) 195.67, 191.20, 161.94, 140.51, 138.18, 130.13, 126.39, 126.18, 123.93, 123.15, 122.47, 121.00, 120.52, 119.00, 117.65, 109.32, 108.72, 108.59, 96.60, 37.70, 29.08, 27.84, 19.80, 13.82, 13.72 (Figure 19.C).

A


CML1
%Transmittance
Wavenumber
1774.976 0.000
1898.537 52.220
3048.921 84.723
2933.382 38.283
2988.766 -1.165
1023.245 161.460
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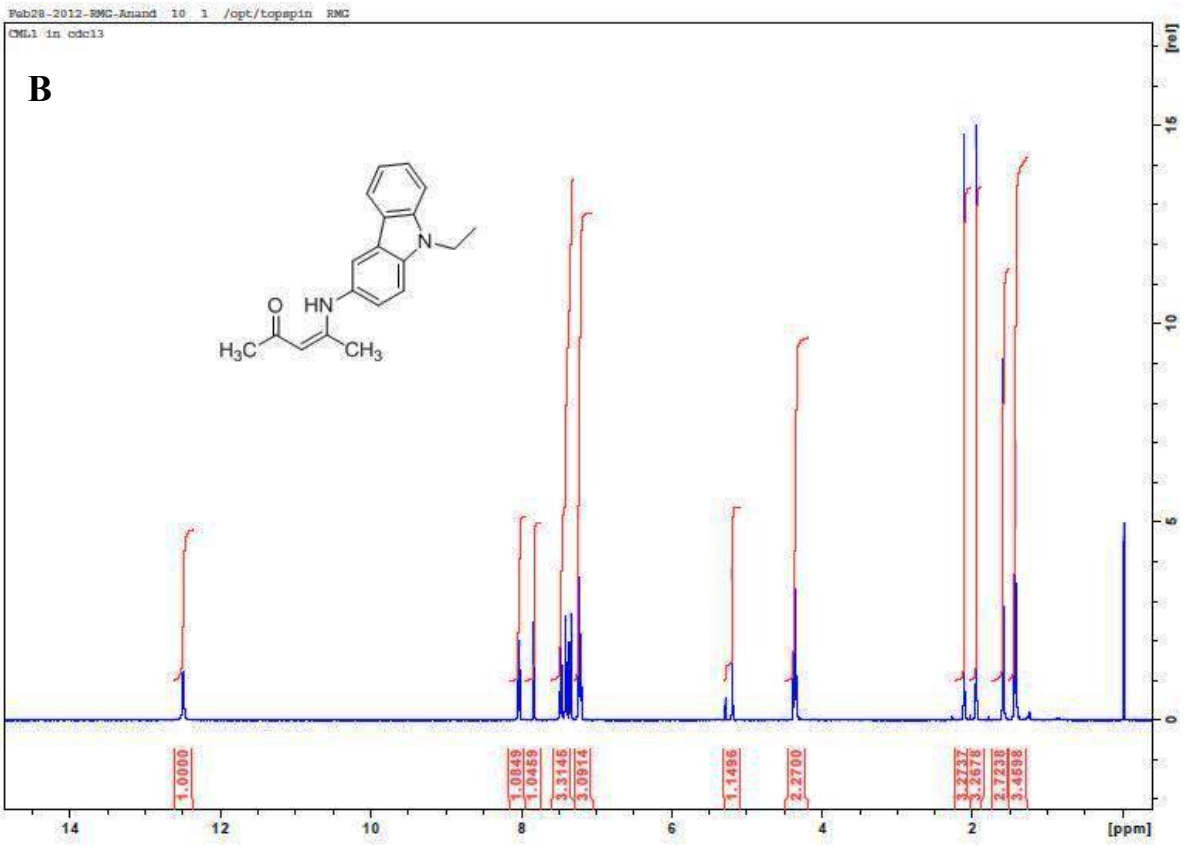
1180.202 154.471
1232.602 194.003
745.719
1278.119 266.522
1562.666 184.155

B


Feb28-2012-RMG-Anand 10 1 /opt/topspin RMG
CML1 in cdcl3
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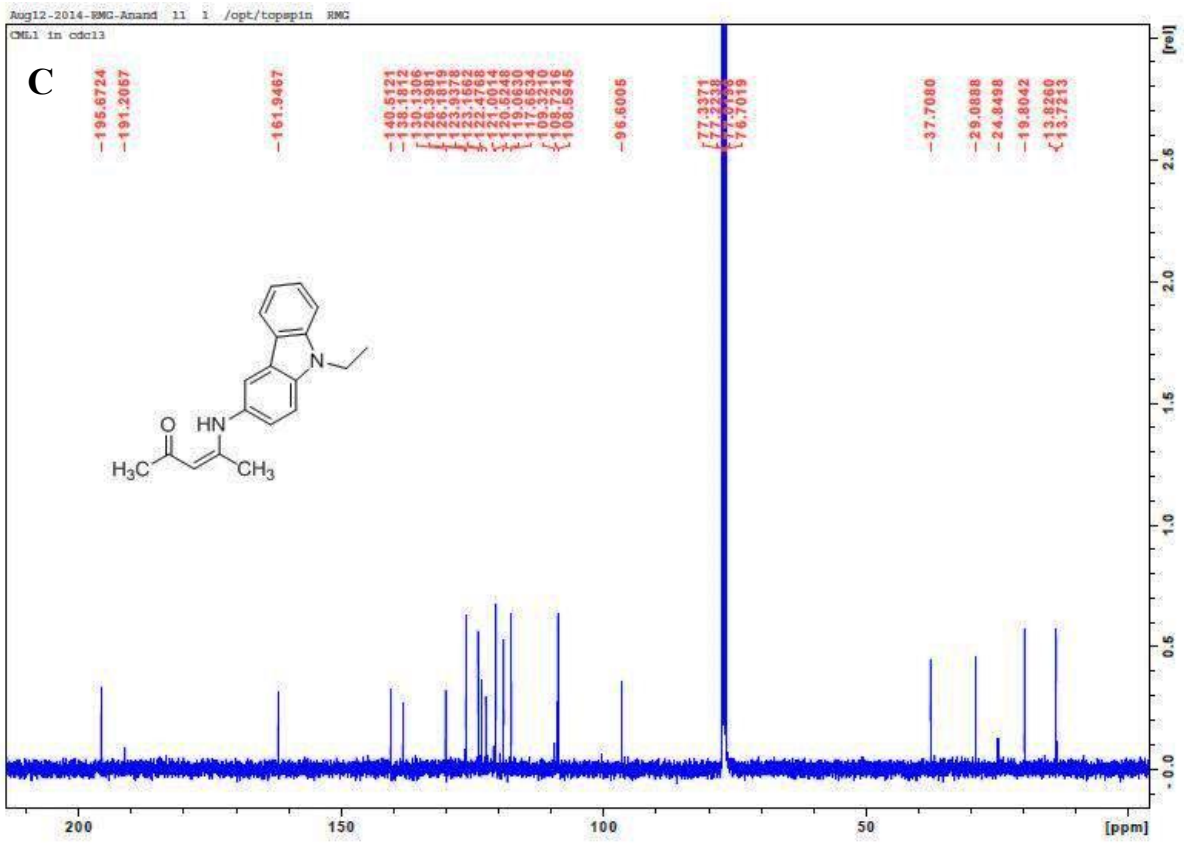

Figure 18: Characterization of the novel (Z)-4-((9- ethyl-9H-carbazol-3-yl)amino)pent-3-en-2-one (ECAP) using A) IR, B) ^{1}H-NMR and C) ^{13}C-NMR spectrums.

3.1.2. Cell viability assay

The MTT assay was used to determine the anti-proliferative effect of ECAP on the A549 lung cancer cells. An IC_{50} value of 0.565 µg/ml at 95% confidence interval was calculated (Figure 20) and subsequently used in all assays.

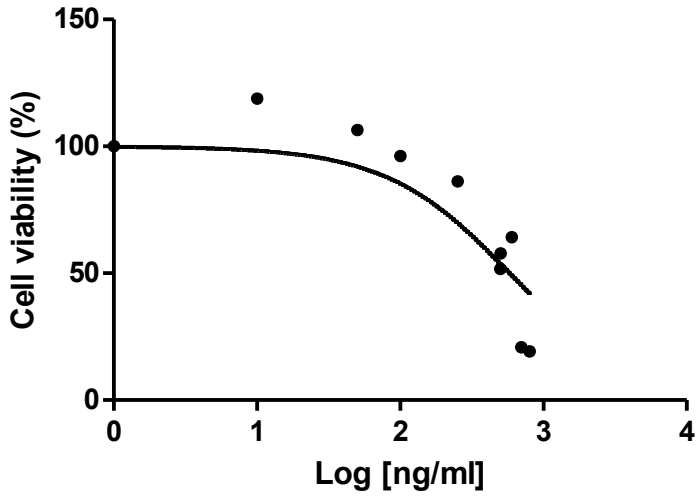

Figure 19: Dose-response curve showing cytotoxic effect of ECAP on A549 lung cancer cell line after 24 h treatment.

Figure 2 demonstrated a decrease in cell viability with increasing concentration.

3.1.3. Lipid peroxidation assay for quantification of malondialdehyde (MDA).

Lipid peroxidation, as measured by the MDA concentration, was significantly increased in ECAP treated cells compared to the control (0.305 ± 0.00490 μM vs 0.190 ± 0.007479 μM (control)) at 95% confident interval (Figure 21).

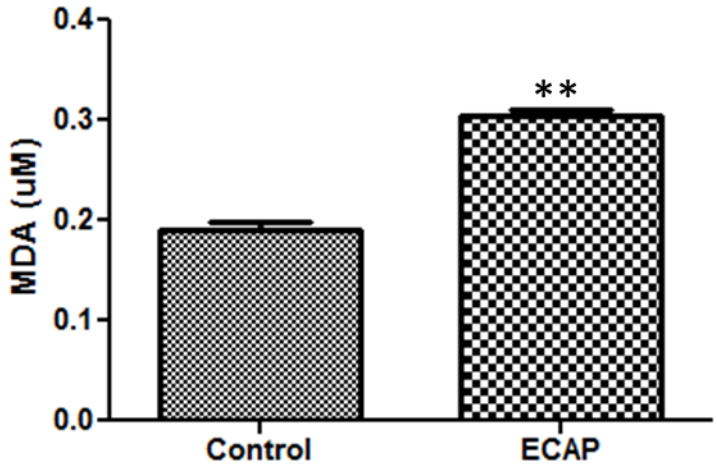

Figure 20: The effect of ECAP on lipid peroxidation on A549 lung cancer cells after 24 h incubation. ($p < 0.0002$) [*** significance compared to the control, number of replicates: $n \geq 3$]

3.1.4. DNA damage

DNA damage was assessed using the comet assay. ECAP significantly increased the length of comet tails in treated cells compared to the control with 81.80 ± 1.19 µm vs 68.20 ± 1.61 µm (control) (Figure 22).

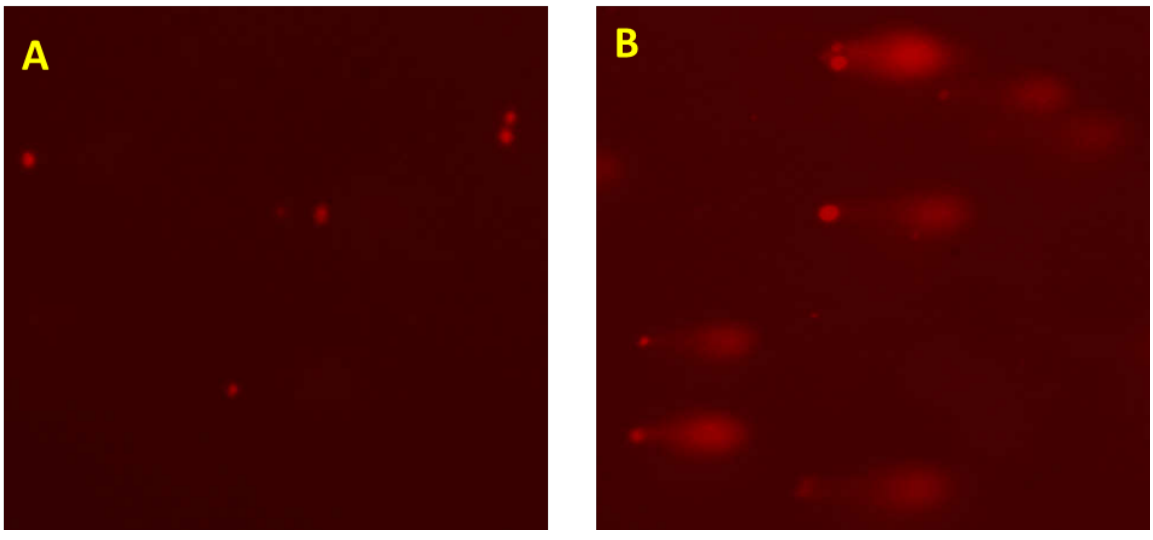

Figure 21: Comet assay showing DNA damage in A549 cells after 24 h treatment with A) Control and B) ECAP. ($p < 0.0001$)

3.1.5. Western blot analysis

Western blotting was used to determine apoptotic proteins induced by ECAP in A549 lung cancer cells. Nrf2/Keap1 system regulates the expression of proteins such as SOD and heat shock proteins which are involved in the cellular antioxidant and anti-inflammatory defense (Petri *et al.*, 2012). ECAP significantly down regulated the expression of Nrf2 ($p < 0.02$*) resulting in a subsequent down regulation of Hsp70 ($p < 0.02$*) and SOD ($p < 0.01$**) (Figure 23). ECAP was further observed to activate the expression of pro-apoptotic proteins such as p53 ($p < 0.09$) and Bax ($p < 0.25$) and down regulated the anti-apoptotic Bcl-2 ($p < 0.0006$***) (Figure 23).

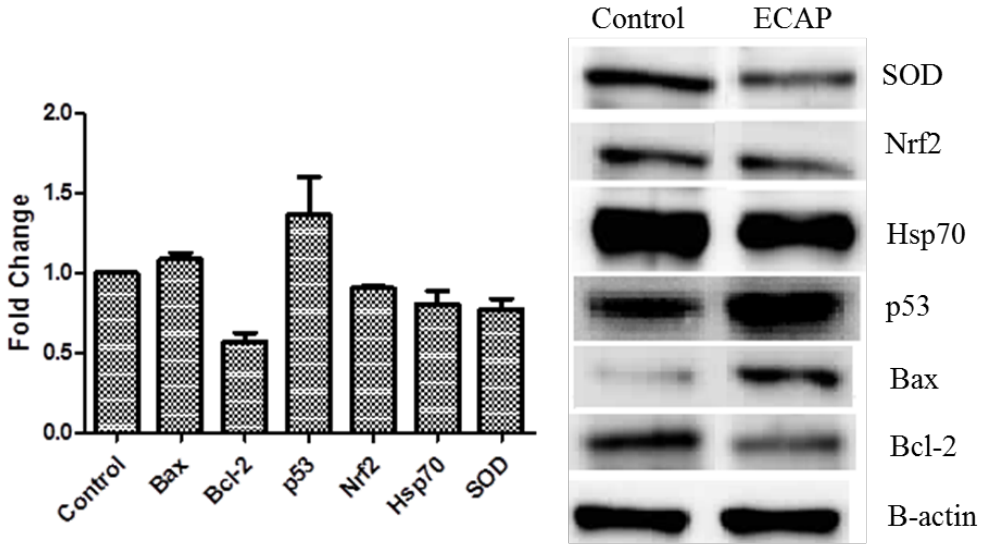

Figure 22: The effect of ECAP on the expression of Bax, Bcl-2, p53, Nrf2, Hsp70 and SOD.

Western blot analysis demonstrated an increase in band density of p53 and Bax compared to the control, while a decrease in band density was observed for Bcl-2, Hsp70, SOD and Nrf2 compared to the control.

3.1.6. Caspase-3/7, 8, 9 and ATP activities

Caspases are ATP dependent enzymes that promote apoptosis. The effects of ECAP on the activity of caspases and ATP levels were measured in A549 lung cancer cells (Table 1)

Table 1: Caspases and ATP levels in ECAP treated A549 cells after 24hr treatment.

	Mean ± SD (RLU × 10^4)			
	Control	ECAP	Fold change	*p*-value
Caspase-3/7	1.831 ± 394.7	2.895 ± 1265	1.471	0.0029**
Caspase-8	67.220 ± 2927	76.930 ± 5513	1.144	0.0001***
Caspase-9	34.440 ± 10760	54.980 ± 6295	1.601	0.0001***
ATP	305.100 ± 35420	272.500 ± 42010	0.894	0.0040**

*denotes statistically significance with respect to the control and uncertainties represent standard deviation (SD) from the means. Number of replicates: $n \geq 3$. ** $p < 0.001$, *** $p < 0.0001$

Table one demonstrated a significant increase in caspase and ATP activity of ECAP treated A549 cells compared to the control. Caspase-9 had a higher fold change compared to caspase-8.

3.1.7. Annexin-V-Fluos assay

Translocation of the membrane PS is one of the markers of the early stages of apoptosis. Figure 24 demonstrated that treatment with ECAP significantly induced apoptosis in A549 lung cancer cells (50.4 ± 0.44%) compared to the control (25.1 ± 0.34%).

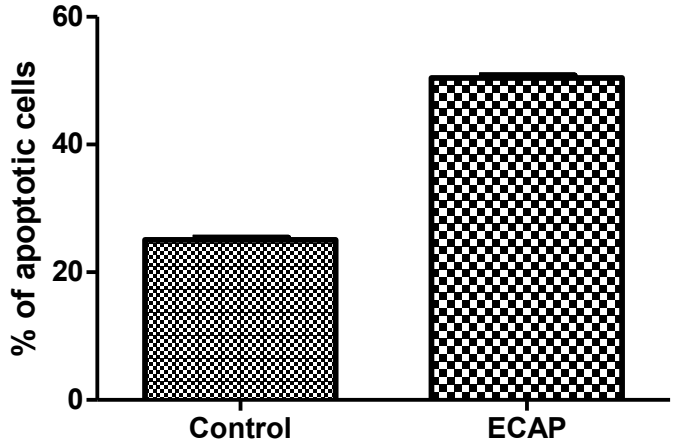

Figure 23: The effect of ECAP on the induction of apoptosis of A549 cells after 24 h treatment. [($p < 0.0001$), *** significance compared to the control and number of replicates: $n \geq 3$]

CHAPTER FOUR

4.1 DISCUSSION

The results of this study demonstrated the ability of ECAP to induce reduction in cell viability, to elevate lipid peroxidation, to induce DNA damage as well as its effect on PS externalization in A549 lung cancer cells. Moreover ECAP was observed to induce reduction in the expression of Nrf2, SOD, Hsp70 and Bcl-2 and overexpression of p53 and Bax in treated cells. Activation of ATP, caspase-3/7, -8 and -9 activity was also observed in the presence of ECAP.

Based on their reported biological properties, an increased research interest has been driven towards studying the anticancer properties of carbazoles and their synthetic analogs. The cytotoxic activity of carbazoles and some of their analogs has been previously demonstrated on various cancer cell lines including lung, leukaemia and pancreatic cells (Mohan *et al.*, 2013, Laronze *et al.*, 2005, Mandal *et al.*, 2012). The results of this study demonstrated the ability of the novel synthesized carbazole compound to induce cytotoxicity on A549 lung cancer cells (Figure 20).

Anticancer therapies such as chemotherapy and radiotherapy make use of ROS overproduction to inhibit the proliferation of cancer cells (Renschler, 2004). The TBARS assay data demonstrated that ECAP also induces ROS production, whereby a significant elevation of MDA concentration corresponded with an increase in lipid peroxidation (Figure 21).

Lungs are highly organized organs, which play an important role in mammalian respiration.

Lungs have elevated levels of Nrf2, which makes them resistant to ROS generated during gas exchange. Nrf2 is the primary antioxidant response regulatory protein which translocate to the nucleus upon oxidative damage, where it promote transcription of antioxidant proteins such as SOD as well as the transcription of Hsp70 (Nguyen *et al.*, 2009, Zoja *et al.*, 2014, Kansanen *et al.*, 2013, Velichkova and Hasson, 2005). Elevated Nrf2 levels have been reported in various cancer cell lines including A549 lung cancer cells. Nrf2 overexpression in lung cancer cells account for their resistance and metastasis thus making lung cancer therapy difficult.

There is a need for development of novel anticancer compounds that are able to reduce Nrf2 expression in lung cancer. Therefore, the effect of ECAP on Nrf2 expression was determined. Western blot data demonstrated a reduction in Nrf2 expression in the presence of ECAP (Figure 23). This might possibly mean that ECAP induced the activity of Keap-1, which then targeted Nrf2 for ubiquitin-mediated degradation. Hence the observed down-regulation of the expression of Nrf2 in the presence of ECAP could then explain the reduced levels of the expressed Hsp70 and SOD in A549 lung cancer cells (Figure 23). This resulted in the observed induced lipid peroxidation, which was due to ROS overproduction as measured by elevated MDA concentration.

Down regulation of antioxidant proteins is associated with ROS overproduction and induction of chronic oxidative damage on biomolecules such as DNA, proteins and lipids (Li *et al.*, 2011). Therefore in addition to the detected high levels of MDA, ECAP was observed to significantly induce DNA damage in A549 cell as demonstrated by high intensity and longer lengths of comet tails (Figure 22).

The observed chronic oxidative DNA damage in comet assay could have resulted in the increased overexpression of serine 46 phosphorylated p53 in ECAP treated A549 lung cancer cells (Figure 23). This is simply because it has been reported that DNA damage can activate p53 protein phosphorylation at serine 46 residue resulting in cell cycle arrest and subsequent DNA repair (Bai and Zhu, 2006). The western blot data further demonstrated Bax activation and down-regulation of Bcl-2 expression in the presence of ECAP (Figure 23). This could mean DNA damage was beyond repair, for this reason the activated p53 induced cell death through activation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins. As a result this makes ROS-mediated DNA damage to be an essential therapeutic target for cancer treatment.

Bax activation is reported to induce mitochondrial polymerization thereby disrupting the electron transport chain (ETC). Therefore, ATP levels were measured to study the effect of the compound on mitochondrial integrity.

The previous work by Roy and co-workers (2005) demonstrated mehanine, the carbazole alkaloid to depolarize the mitochondrial membrane of U937 cells resulting in 60-40% reduction on cellular ATP level compared to the control. In light of this discovery, a significant 0.894 fold decrease in cellular ATP level with respect to the control in A549 lung cancer cells was observed in the presence of ECAP (Table 1). This could mean that just like mahanine, the novel carbazole compound depolarized the mitochondrial membrane of A549 lung cancer cells resulting in cytochrome c release and the subsequent activation of caspase-9.

So far the data demonstrated the cytotoxicity of ECAP in A549 lung cancer cells to be associated with its ability to induce ROS overproduction, which resulted in lipid peroxidation and DNA damage, thus activating the expression of tumour suppressor proteins such as p53. Accordingly, in order to further stipulate the mechanism of action of this novel carbazole compound, luminometry was used to assess the effect ECAP on the activity of the caspases. Caspases are the excussioner molecules involved in both the extrinsic and the mitochondrial dependent pathways of apoptosis.

Mahanine, a carbazole alkaloid isolated from *Micromelum minutum* has been reported to induce apoptosis through a mitochondrial dependent pathway (Kagan et al., 2002, Roy et al., 2005), therefore a significant higher fold change of 1.601 (caspase-9) compared to that of 1.144 (caspase-8) demonstrated ECAP to also mainly execute apoptosis of lung cancer cells through the intrinsic-mitochondrial pathway (Table 1).

ECAP treated cells also demonstrated an induced caspase-3/7 activity (1.471 fold change). The caspase activity data might be due to that activation of executioner caspases activated the initiator caspases-3/7 resulting in down-stream cleavage of proteins such as poly(ADP-ribose) polymerase (PARP) thus inducing apoptosis of A549 lung cancer cells.

As mentioned earlier, PS externalization is an early marker of apoptosis, therefore a higher percentage of PS detected in treated cells compared to the control during Annexin V staining further demonstrated the anticancer properties of ECAP to be associated with its ability to induce apoptosis of A549 lung cancer cells. Up to this far it is evident that this novel carbazole compound comprises of unique properties rendering ECAP as a potential antiproliferative agent towards A549 lung cancer cells. Therefore both the caspase activity and Annexin-V data could mean that ECAP activated an intrinsic mitochondrial-mediated apoptosis of cultured lung cancer cells through activation of executioner the Caspase-9 which activated the initiator caspases-3/7 thereby resulting in down-stream cleavage of proteins such as PARP.

The results of this study demonstrated that the cytotoxic properties of (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one could possibly be associated with its ability to down regulate the expression of antioxidant defense proteins (Nrf2 and SOD) and Hsp70 thus activating ROS overproduction. This ROS overproduction induced DNA damage, which up-regulated the expression of tumor suppressor proteins such as p53.

p53 activation down-regulated the expression of Bcl-2 thus activating the expression of Bax. Bax activation therefore resulted in mitochondrial polymerization and the release of cytochrome c as evidenced by a decrease in cellular ATP levels. The released cytochrome c then activated caspase-9, which subsequently resulted in activation of caspae-3/7 thus inducing a mitochondrial-mediated apoptosis of A549 lung cancer cells.

However this was an *in vitro* study, therefore more work still need to be done in order to authenticate the cytotoxic pathway of ECAP on A549 lung cancer cells. Therefore it might be too early to conclude that ECAP induced cytotoxicity on A549 lung cancer cells through an apoptotic pathway. As a result in order to prove that ECAP induced apoptosis of cultured lung cancer cells more experiments such a Jc-1 stain and Hoechst stain should be carried out. It has been reported that one of the major challenges associated with development of anti-cancer drugs is the ability to develop highly effective drugs which are specific for cancer cells and have a little or no side effects on normal mammalian cells.

Cancer cells are characterized by mitochondrial dysfunction and increased metabolic activity making cancer cells to have higher ROS level compared to non-cancer cells. This excessive ROS production can result in increased cancer cell sensitivity thus functioning as tumour suppressors. Therefore the ability of ECAP to suppress Nrf2, SOD and Hsp70 activity in A549 lung cancer cells can result in ROS overproduction rendering this novel compound to be more specific to cancer cells.

As mentioned earlier in the literature review carbazole compounds and their derivatives are able inhibit cancer proliferation through DNA intercalation and inhibition of DNA topoisomerase II activity (Nandy *et al.*, 2014). However, both cancer and non-cancer cells have DNA and DNA topoisomerase II, therefore ECAP might induce DNA intercalation and inhibit DNA topoisomerase II in both cancer and non-cancer cells.

Thus the specificity of ECAP should be determined using different cancer cell lines as well as using non-cancer cells.

There is also need to determine the effect of ECAP on epigenetic changes and microRNA expression on lung cancer cells. After a proper *in vitro* characterization of ECAP on A549 lung cancer cells and other cancer and non-cancer cell lines, animal models should then be introduced for further characterization of the effects of this novel synthesized carbazole compound before it goes into clinical trials.

CHAPTER FIVE

5.1. CONCLUSION

The study demonstrated the anticancer properties of ECAP to be due to its ability to induce p53 mediated apoptosis on lung cancer cells through activation of oxidative stress, hence down-regulating the expression of anti-apoptotic proteins and antioxidant defense proteins. This resulted in DNA damage and subsequent up-regulation of tumour suppressor genes, pro-apoptotic proteins and apoptosis executioner molecules (Figure 25).

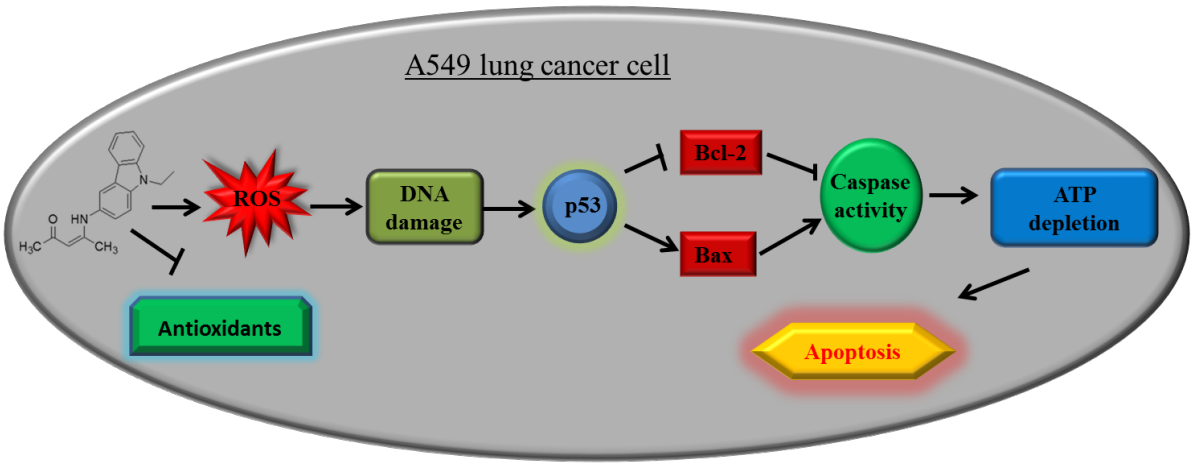

Figure 24: A schematic summary of apoptotic pathway of a novel cabazole compound (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one on A549 lung cancer cells.

This work therefore show the novel carbazole compound (Z)-4-[9-ethyl-9aH-carbazol-3-yl) amino] pent-3-en-2-one to possess potential pharmaceutical properties as an alternative treatment for lung cancer. However, since this was an *in vitro* study, more work still need to be done to further characterize this novel compound and to fill in the gaps in the postulated pathway of action, as well as to analyze its specificity.

Future work will include monitoring the effect of methylation of ECAP on epigenetic changes and its effect on histones and microRNA expression. Its specificity will also need be studied using different cancer and non-cancer cell lines. After a proper *in vitro* characterization of ECAP, animal models can then be introduced to help to further characterize this novel compound to get a broader picture of its mode of action and its side effects. The structure of the compound will also be modified as necessary to improve its effectiveness.

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SUPPLEMENTARY MATERIAL

S1: These supplementary materials represent data for figure 6: Western blots showing the effect of ECAP on the expression of Bax, Bcl-2, p53, Nrf2, Hsp70 and SOD.

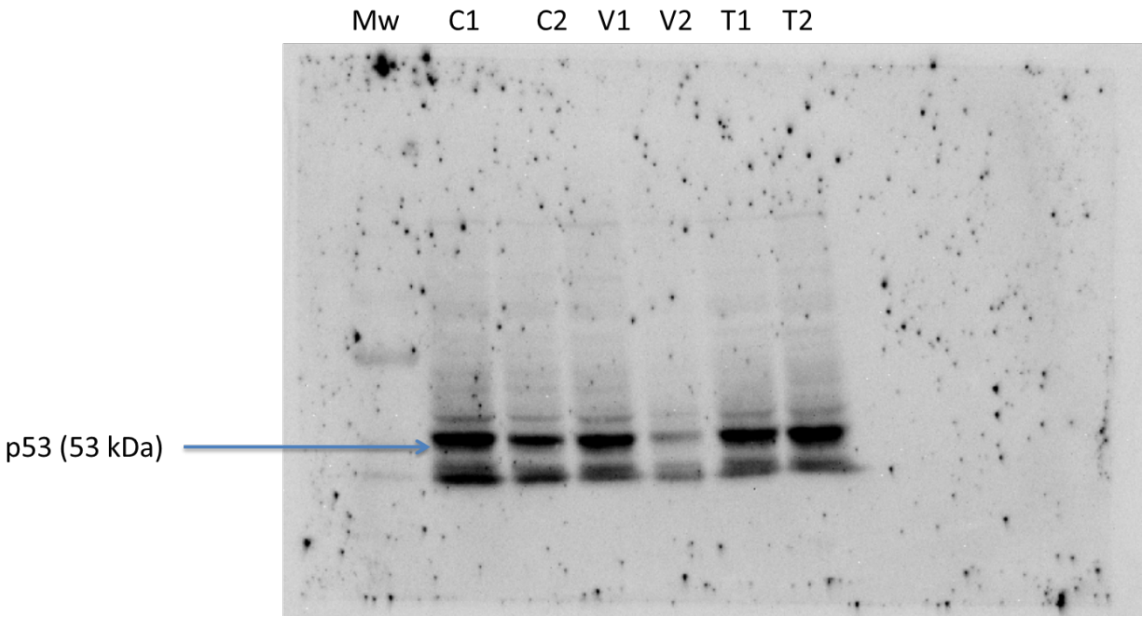
Mw: Molecular marker

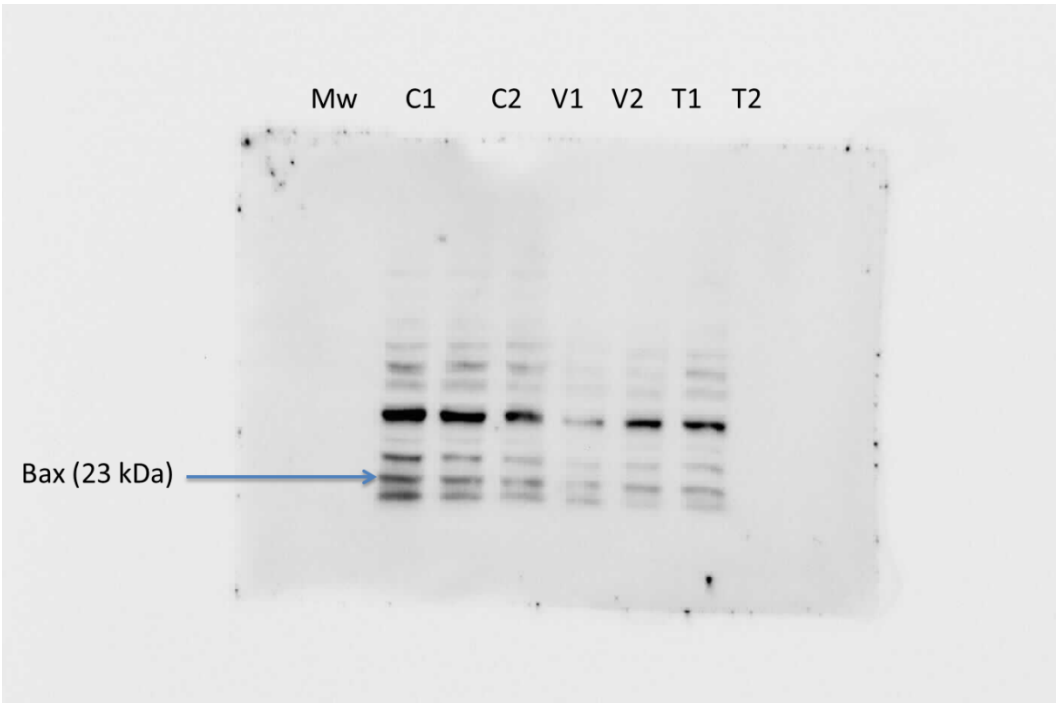
C1 and C2: Controls

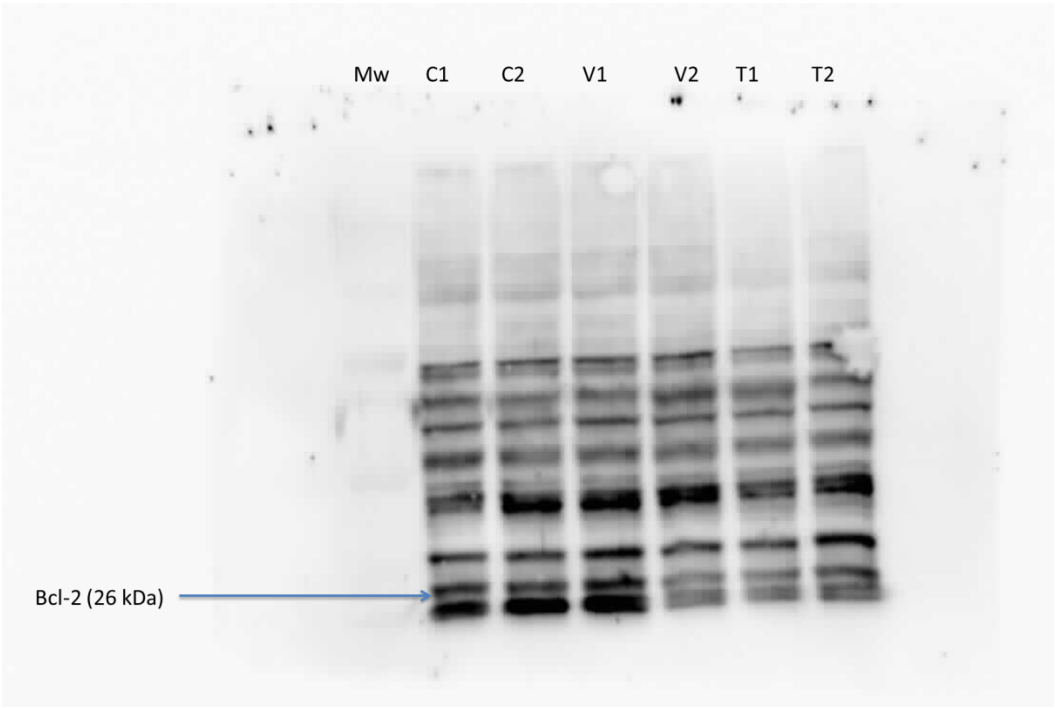
V1 and V2: Vehicle controls

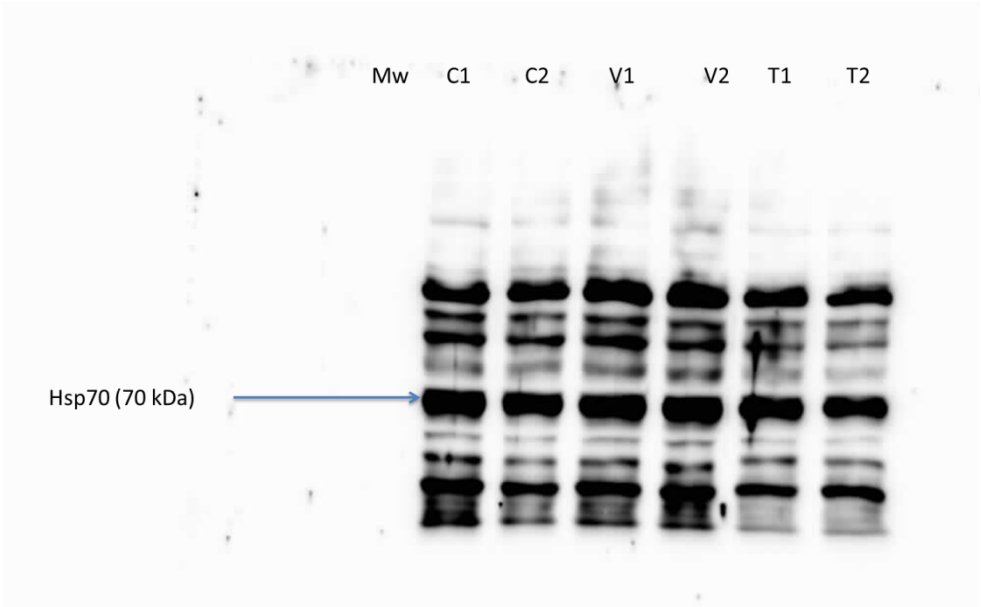
T1 and T2: treatments with ECAP

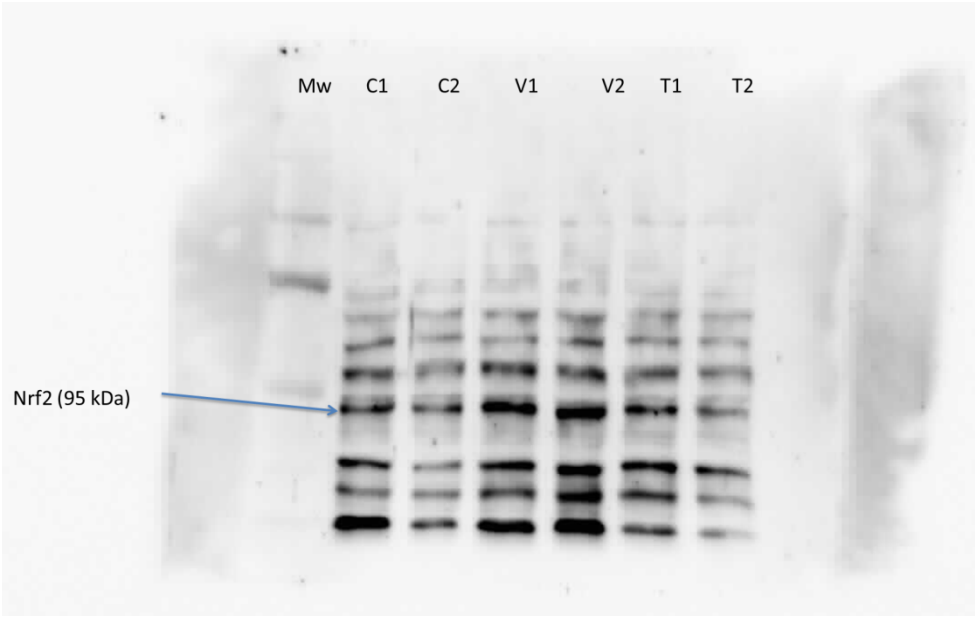
For the basis of data presentation in the results section of the paper, as well as on this dissertation, data was only presented for vehicle control and treatments.

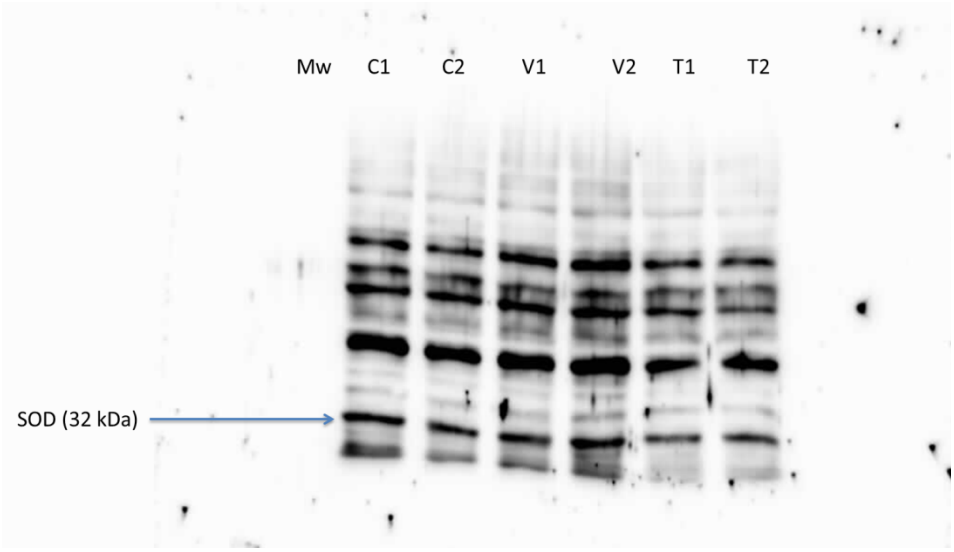



Mw
C1
C2
V1
V2
T1
T2
Bax (23 kDa)

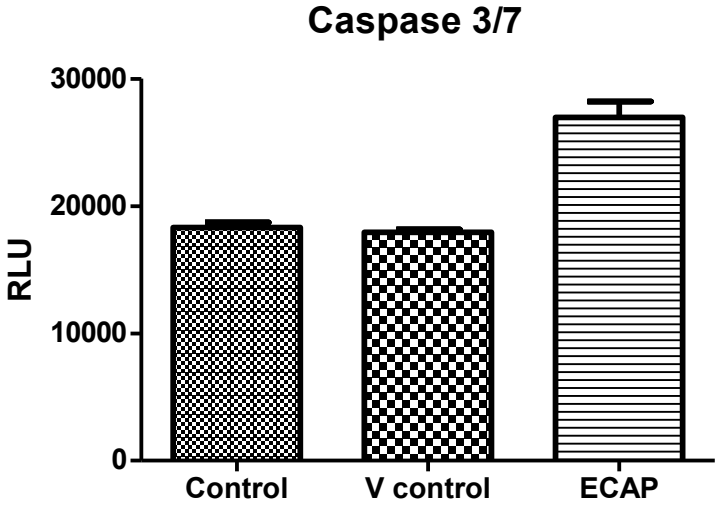

Mw
C1
C2
V1
V2
T1
T2
Bcl-2 (26 kDa)

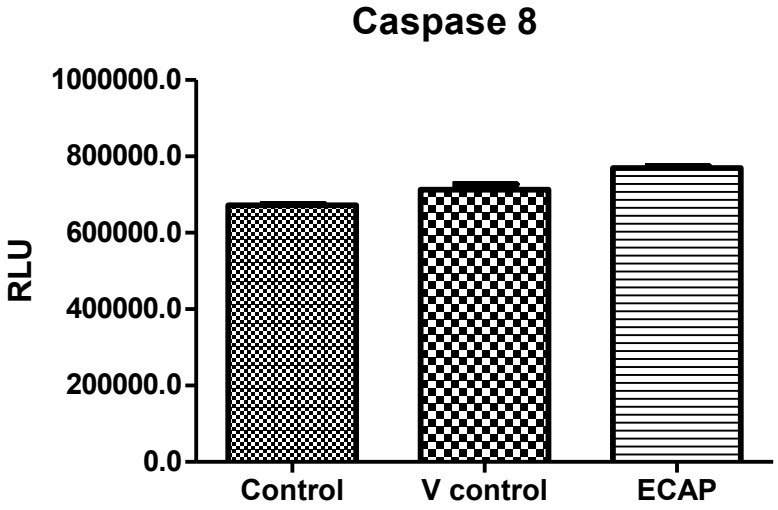

Mw
C1
C2
V1
V2
T1
T2
Hsp70 (70 kDa)

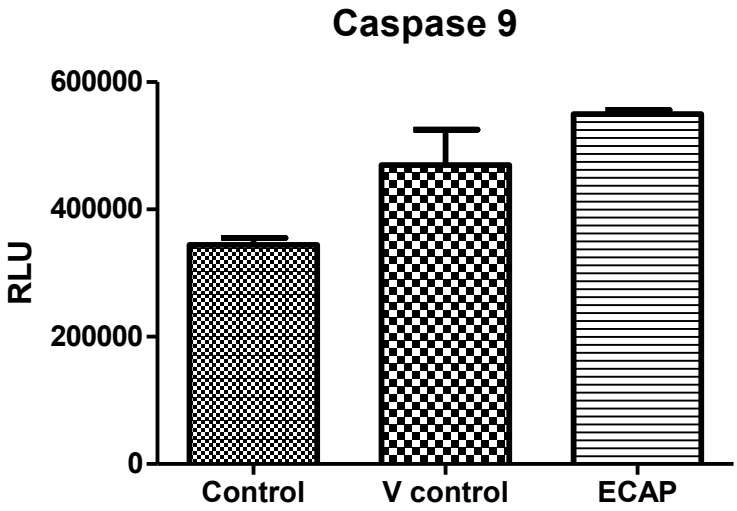

Mw
C1
C2
V1
V2
T1
T2
Nrf2 (95 kDa)

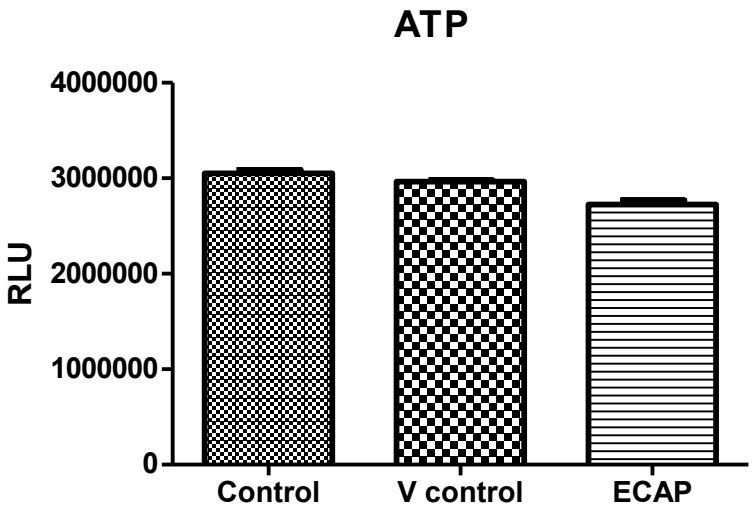

Mw
C1
C2
V1
V2
T1
T2
SOD (32 kDa)

S2: Represent the luminometry data






Caspase 9
600000
400000
200000
0
RLU
Control
V control
ECAP


ATP
4000000
3000000
2000000
1000000
0
RLU
Control
V control
ECAP